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UNIVERSITY OF ALBERTA

STRUCTURE AND FUNCTION STUDIES OF
Manduca sexta LIPID TRANSFER PARTICLE



BY

BRENDA J. BLACKLOCK

A thesis submitted to the Faculty of Graduate Studies and Research in partial
fulfillment of the requirements for the degree of Doctor of Philosophy

Department of Biochemistry

Edmonton, Alberta

Spring 1994



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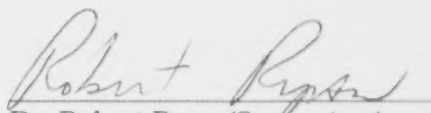
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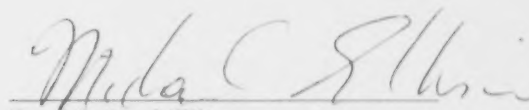
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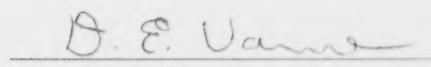
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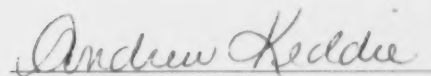
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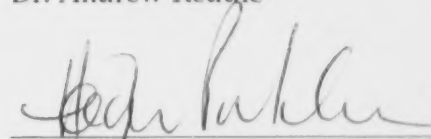
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Sincerely,

Robert O. Ryan

Dedication

To my parents,
Murray and Eunice Blacklock,
for giving me the freedom to follow my dreams,
and
to Rob Minto, for helping me realize them in so many ways.

Abstract

Insect hemolymph lipid transfer particle (LTP) is intimately involved in lipophorin metabolism. This lipoprotein is thought to be responsible for lipophorin subspecies interconversion and the transfer of lipid into lipophorin from tissues. LTP is unique among lipid transfer catalysts. It is a multimeric complex of lipid and three apoproteins, apoLTP-I, apoLTP-II, and apoLTP-III, with a particle molecular weight of over 1×10^6 . LTP possesses a unique morphology with a quasispherical head region and a hinged tail region as demonstrated by electron microscopy. *In vitro* studies have shown that LTP catalyzes the net vectorial transfer of lipid between lipoproteins.

The studies described in this dissertation were aimed at further understanding structure, function, and activity relationships of *Manduca sexta* LTP. The mechanism of action of LTP was investigated utilizing a novel system in which lipoprotein substrates of LTP were covalently bound to physically separated matrices and the transfer of lipid between them determined. These studies demonstrated that LTP utilizes a carrier-mediated mechanism of action in lipid transfer. Polyclonal antibodies were raised against each LTP apoprotein and utilized to demonstrate the immunological uniqueness of the apoproteins and a role for apoLTP-II in lipid transfer. Immunoprecipitation experiments examined the spatial organization of the lipid transfer particle and demonstrated that apoLTP-I and apoLTP-II are exposed on the surface of the particle while apoLTP-III appears to be associated with lipid and, in some cases, is inaccessible to antibody binding. Further experimentation revealed the ability of apoLTP-III to dissociate from the complex and electron microscopic studies suggested that lipid is associated with the head region. Taken together, these results suggest that apoLTP-III also resides in the head region of the particle. Denaturation studies were undertaken through biochemical and biophysical approaches to examine the stability of the complex and the nature of apoprotein interaction. The isolation of apoLTP-I and apoLTP-II was achieved by detergent denaturing gel filtration chromatography. The work presented in this thesis provides insight into the mechanism of LTP lipid transfer, the apoproteins involved, and structural characteristics which may play a role in lipid transfer.

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List of Abbreviations

ABTS	2,2'-azino-di-[3-ethylbenzthiazoline] sulfonate
AKH	adipokinetic hormone
apo A-1	apolipoprotein A-1
apo A-2	apolipoprotein A-2
apo B	apolipoprotein B
apoLp-I, -II, -III	apolipophorin-I, -II, -III
apoLTP-I, -II, -III	lipid transfer particle apoprotein-I, -II, -III
CAPS	3-[cyclohexylamino]-1-propanesulfonic acid
CD	circular dichroism
CE	cholesteryl ester
CETP	cholesteryl ester transfer protein
DAG	diacylglycerol
DEAE	diethylaminoethyl
DFP	diisopropylfluorophosphate
DTT	dithiothreitol
ELISA	enzyme-linked immunosorbent assay
ESR	electron spin resonance
HDL	high density lipoprotein
HDLp-A	high density lipophorin-adult
HDLp-L	high density lipophorin-larval
HDLp-W1	high density lipophorin-prepupal 1
HDLp-W2	high density lipophorin-prepupal 2
IgG	immunoglobulin class G
Iodo-gen	[1,3,4,6-tetrachloro]-3 α ,6 α -diphenylglycoluril
LDL	low density lipoprotein
LDLp	low density lipophorin
Lp-I	lipophorin loading intermediate
LTP	lipid transfer particle
MTP	microsomal triacylglycerol transfer protein complex
NMR	nuclear magnetic resonance
NP-40	Nonidet-40 detergent
PBS	phosphate-buffered saline
PDI	protein disulfide isomerase

PL	phospholipid
PLTP	phospholipid transfer protein
PMSF	phenylmethanesulfonyl fluoride
PVDF	polyvinylidene difluoride
PAGE	polyacrylamide gel electrophoresis
SDS	sodium dodecyl sulfate
TBS	Tris-buffered saline
TTBS	Tris-buffered saline/0.2 % Tween-20
TAG	triacylglycerol
VHDL	very high density lipoprotein
VHDLp-E	very high density lipoprotein-egg
VLDL	very low density lipoprotein

A, Ala	alanine
R, Arg	arginine
N, Asn	asparagine
D, Asp	aspartate
C, Cys	cysteine
E, Glu	glutamate
Q, Gln	glutamine
G, Gly	glycine
H, His	histidine
I, Ile	isoleucine
L, Leu	leucine
K, Lys	lysine
M, Met	methionine
F, Phe	phenylalanine
P, Pro	proline
S, Ser	serine
T, Thr	threonine
Y, Tyr	tyrosine
W, Trp	tryptophan
V, Val	valine

CHAPTER 1

INTRODUCTION: LIPID TRANSPORT IN INSECTS

portions of this chapter are from a review:

Blacklock, B. J. and Ryan, R. O. (1994) Hemolymph Lipid Transport, *Insect Biochemistry and Molecular Biology*. in press.

1.1 Introduction.

The need to transport hydrophobic molecules from sites of absorption and storage to sites of utilization through an aqueous bloodstream is encountered by vertebrate and invertebrate animals alike. Many organisms solve this dilemma by assembling complexes of protein and lipid called lipoproteins, in which lipophilic molecules are sequestered from the aqueous environment within a shell of amphipathic proteins and phospholipids. The lipid complement of lipoproteins are modulated by the action of intracellular and plasma lipid transfer catalysts. Lipid transfer catalysts are important in the initial recruitment of lipid by some nascent lipoproteins and in the remodelling of plasma lipoproteins. Mammalian lipoprotein metabolism is complex and involves three classes of lipoprotein particles, very low density, low density, and high density lipoproteins. Two plasma lipid transfer proteins, cholesteryl ester transfer protein and phospholipid transfer protein, function in plasma lipoprotein metabolism while an intracellular transfer protein, microsomal triacylglycerol transfer protein, is thought to be responsible for the assembly of triacylglycerol-rich lipoproteins. In the insect, the lipid transport vehicle is called lipophorin (from Greek *lipos* : lipid and *phoros* : bearing) and the hemolymph lipid transfer catalyst is the lipid transfer particle. Movement of lipid by lipophorin represents a simplified model for general lipid transport.

The transport of lipids through the open circulatory system of the insect has been the subject of research interest for the more than thirty years since lipophorin was discovered. Our knowledge of the mechanisms of the movement of lipids within insect hemolymph has been especially broadened in the last fifteen years as many groups have focussed on two insect models in

particular, the tobacco hornworm, *Manduca sexta*, and the migratory locust, *Locusta migratoria* (for reviews see: Gilbert and Chino, 1974; Chino and Kitazawa, 1981; Chino, 1985; Beenackers *et al.*, 1986; Shapiro *et al.*, 1988; Ryan, 1990; Kanost *et al.*, 1990; Van der Horst, 1990; Law *et al.*, 1992; Blacklock and Ryan, 1993; Van der Horst *et al.*, 1993). These species are important in the study of lipid transport because they represent insects which depend upon lipid as the primary source of energy for long term flight. This dissertation describes structure and function studies of one component of the insect lipid transport system, the lipid transfer particle (LTP). This chapter will review lipoprotein metabolism in the insect and discuss our current knowledge of LTP. The objectives of the work included in this thesis will also be described.

The physiology of the insect is quite different than that of vertebrate organisms. Insects have an open circulatory system in which the hemolymph bathes vital organs and transports digested food and metabolic waste. Circulation of the hemolymph is achieved by a tubular, pulsatile organ, the heart or dorsal vessel. Gas exchange is conducted by a system of multi-branched tubes (trachea and tracheoles) which open directly to external air. The fat body, which acts as both adipose tissue and liver of vertebrates, is dispersed throughout the body, and serves as the major biosynthetic and storage organ. The flight muscle is responsible for flight in the adult and is highly specialized in the utilization of energy for flight. It does not store energy itself and depends on lipophorin-transported lipid from the fat body to function.

1.2 Role of lipophorin in lipid transport.

Upon assimilation of dietary fat into midgut tissue, lipid molecules such as sterols, phospholipids, and glycerolipids must be incorporated into lipophorin particles for transport through the hemolymph. In *M. sexta*, two midgut fatty acid binding proteins appear to have a role in the movement of long-chain fatty acids through midgut intracellular compartments, thus, aiding in the delivery of lipid to hemolymph lipophorin at the surface of the midgut (Benning *et al.*, 1992; Smith *et al.*, 1992). Since midgut tissue lacks the ability to synthesize the requisite apolipoprotein components, it is thought that lipid is transferred from the midgut to pre-existing lipophorin particles resident in the hemolymph. Lipophorin particles then traverse the hemolymph to the fat body, or other sites of utilization, where the lipid components can be discharged. Docking of lipophorin at the fat body is thought to be mediated by a receptor (Tsuchida and Wells, 1990) or binding site (Van Antwerpen *et al.*, 1989; Schulz *et al.*, 1991) which allows the delivery of lipid to the fat body where it is stored until needed for energy.

Commencement of flight in adults of species which rely on lipid as a flight fuel initiates the mobilization of lipid stores from the fat body (Figure 1.1). Carbohydrates are used for energy for the first 30 min of flight (Beenackers *et al.*, 1984) after which lipid becomes the primary source of energy. The exhaustion of carbohydrate resources before the use of lipid stores and the inhibition of mobilization of lipid from the fat body during rest appears to be mediated by the hemolymph disaccharide, trehalose. Trehalose inhibits the *in vitro* loading of lipophorin with lipid from the fat body when

present in physiological concentrations found when the animal is at rest (Lum and Chino, 1990). The target of trehalose action, however, is unknown.

Mobilization of lipid stores is signalled by the release of a peptide hormone, adipokinetic hormone (AKH), from an endocrine gland, the corpus cardiacum (Beenakkers *et al.*, 1985). AKH binds to an adenylate cyclase-coupled receptor at the fat body inducing the conversion of ATP to cAMP. A putative hormone sensitive lipase is activated by cAMP and stored triacylglycerol (TAG) is, ultimately, converted to diacylglycerol (DAG), the transport form of neutral glycerolipid. DAG is subsequently transferred into pre-existing high density lipophorin particles (HDLp-A) at the surface of the fat body in a process facilitated by LTP (Van Heusden and Law, 1989). Concomitant with DAG uptake, several molecules of an exchangeable apolipophorin, apolipophorin-III (apoLp-III), associate with the particle. The resulting larger and less dense low density lipophorin (LDLp), transports DAG necessary for flight from the fat body to the flight muscle. At the flight muscle, lipophorin lipase (Van Huesden *et al.*, 1987a; Van Heusden, 1993) hydrolyzes DAG to glycerol and free fatty acid which is internalized. A flight muscle fatty acid binding protein which may be involved in the uptake or intracellular transport of lipophorin-derived fatty acids has been identified and characterized (Haunerland and Chisholm, 1990; Price *et al.*, 1992). As DAG is removed from LDLp, apoLp-III molecules dissociate from the surface regenerating HDLp-A. HDLp-A then returns to the fat body to be reloaded with DAG, thereby acting as a shuttle of DAG within the hemolymph (Surholt *et al.*, 1991).

A key characteristic of the transport of lipid by lipophorin is the fact that lipophorin is not endocytosed or otherwise internalized at the midgut, fat body tissue, or flight muscle. The advantage of the formation of lipid-loaded

transport vehicles without the need for protein synthesis or lipoprotein assembly is obvious. The lipid mobilization response can be initiated more quickly if particles which can be loaded with lipid already exist in the hemolymph. Physiologically, this is advantageous since lipid-fuelled flight can be undertaken without the requirement for lipophorin biosynthesis. The ability of lipophorin to act as a reusable shuttle of lipid was hypothesized by Chino and coworkers in the early 1980's (Chino and Kitazawa, 1981; Chino, 1985). Convincing evidence for such a mechanism was demonstrated by marked differences in turn-over rates of lipophorin DAG versus protein within the hemolymph of resting moths (Downer and Chino, 1985). Subsequently, numerous studies have provided support for the shuttle hypothesis (Van Heusden *et al.*, 1987b; Surholt *et al.*, 1991; Van Heusden *et al.*, 1991; Gondim *et al.*, 1992). Lipid-depletion by lipase treatment and subsequent reloading with both phospholipid (PL) and DAG *in vitro* and *in vivo* support the shuttle hypothesis (Van Heusden *et al.*, 1991; Gondim *et al.*, 1992; Atella *et al.*, 1992). Differential radiolabelling of HDLp-A and LDLp apoproteins and lipid have demonstrated conclusively, that lipophorin indeed acts as a shuttle of lipid during flight, since apolipophorins had hemolymph half-lives approximately 20-fold greater than DAG during rest and flight (Surholt *et al.*, 1991).

The inherent ability of lipophorin to sequester lipophilic molecules from the aqueous hemolymph makes it an ideal transport vehicle for hydrophobic molecules in general. For example, lipophorins from some insect species, such as *Periplaneta americana* and *L. migratoria*, have high hydrocarbon contents which may be utilized in cuticle formation (Chino and Kitazawa, 1981). Lipophorin has been shown to transport hydrocarbons from oenocytes, the site of hydrocarbon synthesis, to the cuticle where hydrocarbons are deposited as

cuticular wax (Katase and Chino, 1982). Lipophorin also appears to be important in the transport of free fatty acid in hemolymph (Soulages and Wells, 1994). Similarly, lipophorins have been shown to transport steroids such as diet-derived cholesterol (insects do not have the capacity to synthesize their own cholesterol) which is important in steroid hormone production (Chino, 1985). Lipophorins of many species carry carotenoid non-specifically, presumably in the core of the particle (Bergman and Chippendale, 1992; de Kort and Koopmanschap, 1987). The sesquiterpene juvenile hormone, a key regulator of development, is transported in the hemolymph by juvenile hormone binding proteins. One of these is lipophorin, with which juvenile hormone seems to associate through specific apolipophorin-I (apoLp-I) sites (de Kort and Koopmanschap, 1987; de Kort and Koopmanschap, 1989; Okot-Kotber and Prestwich, 1991; King and Tobe, 1992).

In addition to its primary role as a transport vehicle of lipid between midgut, fat body, and flight muscle, lipophorin has also been shown to be involved in the uptake of lipids by the developing oocyte. Early studies demonstrated that *M. sexta* oocytes contain a very high density lipophorin, VHDLp-E, which is derived from HDLp-A (Chino *et al.*, 1977; Kawooya *et al.*, 1988b). An endocytic process which seems to occur in association with the unloading of lipid from HDLp-A, but not degradation of apolipophorins, is involved in uptake of HDLp-A by the oocyte (Kawooya *et al.*, 1988a; Telfer *et al.*, 1991). The process of HDLp-A cellular uptake by the developing oocyte is unique in that it is the only example of the internalization of lipophorin by a tissue while delivering lipid. HDLp-A appears to be selectively endocytosed while LDLp is not taken up by the oocyte. LDLp, however, is the source of the majority of the lipid contained in the oocyte (Kawooya and Law, 1988).

Vitellogenin also contributes to the lipid stores of the egg and is endocytosed in a manner similar to HDLp. Vitellogenin and HDLp have been shown to compete for uptake into *Hyalophora cecropia* yolk spheres suggesting adsorption of these two lipoproteins is mediated by a common receptor (Kulakosky and Telfer, 1990). Chicken follicles have been shown to endocytose very low density lipoprotein (VLDL) and vitellogenin through binding to a common receptor (Stifani *et al.*, 1990; Barber *et al.*, 1991) suggesting an evolutionary link in the process of yolk formation between insect and avian species.

1.3 Lipophorin structure.

The structure of lipophorin has been studied using a variety of biochemical and biophysical techniques and a number of structural models have been presented. Generally, lipophorin particles are composed of both polar (PL and cholesterol) and apolar (DAG and hydrocarbon) lipids and protein. All lipophorins have two integral apolipoproteins; apolipophorin-I and apolipophorin-II (apoLp-I and apoLp-II). One high density lipophorin subspecies (HDLp-A) and LDLp also possess the exchangeable apoLp-III. Lipophorins are commonly thought to possess a hydrophobic core composed of nonpolar lipid constituents surrounded by a monolayer of amphiphathic PL and apolipophorins. A complicating factor which must be accounted for in models of lipophorin structure is its ability to form several distinct subspecies which differ in lipid content and composition and, in some cases, apolipoprotein content. On the basis of the fact that all lipophorins possess one copy each of apoLp-I and apoLp-II, it has been hypothesized that these apolipophorins, plus a small amount of PL, constitute a basic matrix particle.

This particle has the capacity to accept or donate variable amounts of lipid to produce distinct lipophorin subspecies such as those observed in larval, prepupal, pupal, and adult life stages as well as oocytes of *M. sexta* (Ryan, 1990).

Several studies utilizing differential scanning calorimetry, nuclear magnetic resonance (NMR), small angle X-ray scattering and modeling with lipophorins from a number of species have suggested that HDLp particles have a sphere-like morphology with discrete, concentric regions. The surface of HDLp is hydrophilic and is composed of PL and lipid-embedded apoLp-I, apoLp-II, and, in certain cases, apoLp-III. The core region is thought to be comprised of a portion of apoLp-II and hydrophobic lipids such as DAG and hydrocarbon (Katagiri *et al.*, 1985; Katagiri *et al.*, 1987; Katagiri *et al.*, 1991; Soulages and Brenner, 1991; Ryan *et al.*, 1992a). A number of experimental approaches have suggested that DAG partitions between the core and the surface of the lipophorin particle and provides a hydrophobic patch at the surface of the particle with which apoLp-III molecules associate (Kawooya *et al.*, 1991; Wang *et al.*, 1992; Singh *et al.*, 1994). Electron microscopic studies revealed that *M. sexta* HDLp subspecies are not all spherical in morphology. DAG-rich subspecies (HDLp-A) are spherical while those which possess less lipid appear to have a cleft into which lipid may be loaded (Ryan *et al.*, 1992a).

1.4 Lipophorin biosynthesis.

Lipophorin biosynthesis has been the object of some controversy over the past few years. The use of different insect systems and experimental techniques has resulted in two models of lipophorin biosynthesis. The fat body has been clearly demonstrated to be the site of lipophorin biosynthesis and in

this way acts analogously to the mammalian liver (Prasad *et al.*, 1986a; Capurro and de Bianchi 1990b; Shelby and Chippendale, 1991; Weers *et al.*, 1992; Weers *et al.*, 1993). Prasad *et al.* (1986a) suggested that *M. sexta* lipophorin is synthesized and secreted from fat body as a nascent VHDLP containing apoLp-I and apoLp-II and PL but very little DAG. DAG is thought to be acquired by the nascent particle from dietary lipid at the midgut through the action of LTP (Prasad *et al.*, 1986a). Subsequent studies in *Musca domestica*, *Diatraea grandiosella* and *L. migratoria*, suggested that lipophorin is assembled and secreted from the fat body possessing its full complement of DAG (Capurro and de Bianchi, 1990b; Shelby and Chippendale, 1991; Weers *et al.*, 1992). Pulse chase experiments demonstrated that, while apoLp-I is approximately three times larger than apoLp-II, they are simultaneously secreted from the fat body and coimmunoprecipitate from fat body homogenates. The protein moiety of lipophorin was shown to arise from a single polypeptide chain, proapolipophorin, which, during the assembly and secretion of lipophorin particles, is cleaved into apoLp-I and apoLp-II (Weers *et al.*, 1993). A deduced amino acid sequence of proapolipophorin has been reported to have similarity with a fragment of human apolipoprotein B-100 precursor suggesting an evolutionary link between the integral apolipoproteins of insects and mammals (Van der Horst *et al.*, 1993).

1.5 Lipid transfer particle.

During the transition of *M. sexta* larvae to the pupal life stage, the lipophorin present in the hemolymph undergoes significant changes in density and lipid content and composition without the biosynthesis of new apoproteins

or the intake of additional lipids. The larval form of lipophorin, HDLp-L, is converted to HDLp-W2, within 24 hours of cessation of feeding, through the loss of DAG and hydrocarbon. HDLp-W2 is then converted to the lower density lipophorin, HDLp-W1, within 12 h of the appearance of HDLp-W2 by the acquisition of DAG and PL, apparently from the fat body. HDLp-W1 persists in the hemolymph until pupation when HDLp-P appears (Prasad *et al.*, 1986b). New lipophorin subspecies seem to arise from the retailoring of existing lipophorin in lipid content and composition. The conversions between each of these lipophorin subspecies must be discrete and precisely timed since only one form is present in the hemolymph at each life stage (Prasad *et al.*, 1986b; Ryan *et al.*, 1987). The dynamic nature of lipophorin throughout the various life stages suggests a lipid transfer factor is involved in the interconversion of lipophorin subspecies in larval, prepupal, and adult stages of the life cycle. Evidence for such a lipid transfer factor was first demonstrated when equilibration in apoprotein and lipid content and density of HDLp-W2 and LDLp to produce a lipophorin species intermediate in density to the substrate particles was induced by lipophorin-deficient hemolymph (Ryan *et al.*, 1986a; Ryan *et al.*, 1987). Examination of the intermediate product after short-term incubation revealed the transfer of DAG, PL, and apoLp-III, but not apoLp-I or apoLp-II. Preliminary characterization of the hemolymph lipid transfer factor(s) indicated a proteinaceous moiety was involved since the lipid transfer activity of lipophorin-deficient hemolymph was non-dialyzable and labile to boiling (Ryan *et al.*, 1986a).

The hemolymph factor thought to be responsible in the interconversion of lipophorin subspecies, lipid transfer particle, was isolated from *M. sexta* hemolymph by Ryan *et al.* in 1986 (Ryan *et al.*, 1986b; Ryan *et al.*, 1987). The

initial isolation procedure employed density gradient ultracentrifugation, ammonium sulfate precipitation, and gel filtration chromatography of late fifth instar larval hemolymph. This protocol took advantage of two unique physical characteristics of LTP; its size and density. LTP was originally thought to consist of two apoproteins, apoLTP-I and apoLTP-II (Ryan *et al.*, 1986b), but has subsequently been shown to possess a third apoprotein, apoLTP-III (Ryan *et al.*, 1988c).

The presence of LTP in the hemolymph of other insect species has been demonstrated and appears to reflect the broad range of species possessing lipophorin. LTP has been purified from the hemolymph of the migratory locust, *L. migratoria* (Hirayama and Chino, 1990) and the American cockroach, *P. americana* (Takeuchi and Chino, 1993) as well as *M. sexta*. Purification of LTP from *L. migratoria* and *P. americana* hemolymph employed specific precipitation of lipoproteins under low ionic strength conditions, ion-exchange chromatography, and density gradient ultracentrifugation (Hirayama and Chino, 1990; Takeuchi and Chino, 1993). Lipid transfer activity in the hemolymph of the domestic house fly, *M. domestica*, has also been reported (Capurro and de Bianchi, 1990a). Interestingly, *P. americana* and *M. domestica* do not use lipid as flight fuel supporting the hypothesis that LTP has physiological roles beyond the loading of DAG into LDLp.

1.6 Physiological role of lipid transfer particle.

Many studies have provided clues concerning the *in vivo* importance of the ability of LTP to transfer DAG, PL, and hydrocarbon. The concentration of LTP is relatively constant throughout early larval development and then

increases steadily from the initial stages of the fifth instar until adult emergence (Ryan *et al.*, 1988b). The relative concentrations of LTP throughout development may reflect the importance of lipid transport at various stages of the life cycle. Adult insects which rely on lipid for flight energy may require a higher concentration of LTP than earlier life stages.

The loading of newly synthesized lipophorin particles with DAG from the midgut and fat body, appears to require LTP (Prasad *et al.*, 1986a). As well, LTP has been shown to be involved in the loading of HDLp-A with fat body-derived DAG *in vitro* (Van Heusden and Law, 1989). Incubation of radiolabelled isolated fat body with lipophorin resulted in the incorporation of fat body-derived radiolabelled DAG into lipophorin in the presence and absence of AKH. This transfer was blocked by preincubation with polyclonal anti LTP serum and re-established upon the addition of exogenous LTP. Transfer of radiolabelled lipid from lipophorin to fat body appeared to proceed by a non-LTP catalyzed mechanism suggesting the delivery of dietary lipid from lipophorin to the fat body is independent of LTP (Van Heusden and Law, 1989). Other indirect evidence for the physiological role of LTP in loading lipophorin particles with DAG and PL was obtained in studies where lipophorin was depleted in either DAG or PL by lipase treatment. DAG or PL content was replenished upon *in vitro* incubation with isolated fat body as well as *in vivo* (Kawooya *et al.*, 1991; Van Heusden *et al.*, 1991; Gondim *et al.*, 1992). Radiolabelled PL was also transferred from isolated fat body to lipophorin, apparently by the action of LTP (Atella *et al.*, 1992).

The unloading of HDLp-A in the oocyte appears to be mediated by LTP and a yolk body lipophorin lipase. Van Antwerpen and Law (1992) have recently reported the identification and partial characterization of a *M. sexta*

yolk body lipophorin lipase from yolk body membrane-free lysates. This lipase was shown to be capable of the hydrolysis of HDLp-DAG to free fatty acid and glycerol. LTP has been shown to be capable of transforming HDLp-A to a VHDLp-E-like particle using LDL as a lipid acceptor (Liu and Ryan, 1991). A lipid transfer catalyst with identical electrophoretic and immunological properties to hemolymph LTP was isolated from a *M. sexta* oocyte homogenate. As well, a *M. sexta* oocyte homogenate was shown to induce transformation of HDLp-A to a VHDLp. This activity was inhibited by anti LTP antibodies and the inhibition was relieved by the addition of exogenous LTP (Liu and Ryan, 1991). These results suggest that LTP is involved in the initial unloading of HDLp-A in the oocyte and that lipophorin lipase may not be specific for HDLp-DAG but is, rather, a general glycerolipid lipase. This is supported by the recovery of lipase activity in yolk body lysates and the void volume ($M_r > 80\,000$) of gel filtration chromatography of yolk bodies (Van Antwerpen and Law, 1992) which would also contain yolk body LTP.

1.7 Structural aspects of lipid transfer particle.

LTP has been shown to be a very high density lipoprotein (density = 1.23 g/mL) consisting of three apoproteins, lipid, and carbohydrate. The apoproteins of *M. sexta* LTP; apoLTP-I, $M_r \sim 320\,000$, apoLTP-II, $M_r = 85\,000$, and apoLTP-III, $M_r = 55\,000$ (Ryan *et al.*, 1988c) (Figure 1.2) apparently exist in a stoichiometry of 2 apoLTP-I : 2 apoLTP-II : 1 or 2 apoLTP-III (Ryan *et al.*, 1990b) and are each modified with high mannose oligosaccharides (Ryan *et al.*, 1988c). Non-covalently associated lipid comprises 14% of the particle mass and consists of predominantly PL and DAG with minor contributions from free fatty acid

and TAG (Ryan *et al.*, 1988c). Table 1.1 describes the properties of *M. sexta*, *L. migratoria*, and *P. americana* LTP. Immunological studies demonstrated that the apoproteins of LTP are distinct from lipophorin and vitellogenin apoproteins (Ryan *et al.*, 1988c).

The large size of the LTP complex makes the particle suitable for electron microscopic morphological studies. Observation of LTP by transmission electron microscopy revealed an unusual, asymmetric morphology with two major structural features; a quasispherical head region and a hinged tail region (Ryan *et al.*, 1990b) (Figures 1.3 and 1.4). The hinge of the tail region occurs at the approximate midpoint and is capable of assuming angles from 30 to 180°. A similar unique morphology was observed for *P. americana* LTP (Takeuchi and Chino, 1993). Estimation of the molecular mass of the particle based on dimensional measurements of electron micrographs was 1.4×10^6 daltons. This molecular mass estimate, taken together with estimates of molecular mass of each of the apoproteins based on denaturing sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), suggested the stoichiometry of 2 apoLTP-I : 2 apoLTP-II : 1 or 2 apoLTP-III.

The exposure of LTP apoproteins to the aqueous environment was probed by subjecting the particle to limited trypsin digestion. ApoLTP-I and apoLTP-II were both susceptible to protease degradation while apoLTP-III appeared to be protected under mild conditions. Harsher conditions, where the particle was denatured by SDS, allowed the trypsin digestion of all apoproteins demonstrating that apoLTP-III has trypsin-susceptible sites (Ryan *et al.*, 1990b). These results suggest that either the entire apoLTP-III component or trypsin-susceptible sites of apoLTP-III are buried within the particle. Electron microscopic examination of limited digested LTP revealed an intact particle

indicating that trypsin treatment merely nicked polypeptide chains which retained their higher order structure. As well, trypsin digested LTP floated in density gradient ultracentrifugation in a manner identical to native LTP and retained lipid transfer activity.

Attempts were made to identify the lipid-containing region of the LTP complex. Phospholipase A₂ treatment of LTP resulted in the conversion of phospholipids to their lyso forms but did not affect the SDS-PAGE profile, transfer activity, floatation properties, or morphological characteristics of the particle. Treatment of the particle with SDS caused significant perturbations in the head region as demonstrated by electron microscopy suggesting that lipid may reside in this location (Ryan *et al.*, 1990b).

Biophysical techniques have also been used to examine LTP (Ryan *et al.*, 1990a). LTP was shown to self-aggregate under low ionic strength conditions by analytical ultracentrifugation experiments. The non-self-associated species, found in a higher ionic strength buffer, sedimented with an apparent sedimentation coefficient of 14.8 S. Sedimentation equilibrium experiments indicated a M_r of 887 000 for the particle. Circular dichroism spectroscopic studies and CONTIN analysis of the spectra revealed a secondary structure high in β -sheet content (6 % α -helix, 49 % β -sheet, and 35 % random coil) (Ryan *et al.*, 1990a).

1.8 Catalytic function of lipid transfer particle.

Lipid transfer catalysts can carry out either the net transfer of lipids, the exchange of lipids between donor and acceptor particles, or both. Net transfer

of lipids is characterized by a unidirectional flux such that one substrate becomes enriched whereas exchange is characterized by equivalent bidirectional fluxes of lipid (Helmkamp, 1986). As described below, LTP is capable of both net lipid transfer and exchange.

The catalytic activity of LTP has been tested with a number of insect and human lipoproteins as substrate particles and a variety of lipid species. LTP has been demonstrated to facilitate lipid transfer or exchange among many insect lipophorins including *M. sexta* HDLp-L and LDLp (Ryan *et al.*, 1988c), *Heliothis zea* very high density chromolipoprotein (VHDL) (Ryan *et al.*, 1988b), and *M. domestica*, *L. migratoria*, and *P. americana* HDLp (Capurro and de Bianchi, 1990a; Hirayama and Chino, 1990; Takeuchi and Chino, 1993) without integral apolipoprotein transfer or exchange. As described above, LTP induces the equilibration of substrate *M. sexta* lipophorin density and apoprotein and lipid content (Ryan *et al.*, 1986b; Ryan *et al.*, 1987). *H. zea* VHDL is a tetramer of identical apoproteins which are immunologically distinct from *M. sexta* apolipophorins. Incubation of *M. sexta* [^3H]DAG-HDLp and *H. zea* very high density chromolipoprotein in the presence of LTP resulted in the exchange of DAG rather than its transfer. Reisolation of lipoproteins following incubation revealed that the reaction was limited to DAG exchange, DAG was unmetabolized, and apolipoproteins were not transferred or exchanged (Ryan *et al.*, 1988b).

Incubation of LTP with only LDLp results in lipid and apoprotein disproportionation (Ryan *et al.*, 1990c) and the production of two novel lipophorins; a very low density lipophorin (density = 1.02 g/ml, apolipophorin ratios of 1 apoLp-I : 1 apoLp-II : 21 apoLp-III molecules, 53 % DAG) and a HDLp of higher density than HDLp-A (density = 1.11 g/ml, 1 : 1 : 2 apolipophorin

ratio, 28 % DAG). The origin of the ability of LTP to cause the lipid and apoprotein disproportionation of a population of apparently identical lipophorins is unknown. The LDLp particles used may not be homogeneous but represent a range of lipophorins with slightly differing apoLp-III and DAG contents. The action of LTP on a portion of the LDLp population may cause a change which results in the use of these modified LDLp particles as lipid and apoLp-III donors or acceptors to the remaining nascent LDLp particles (Ryan *et al.*, 1990c).

LTP has also been shown to use human lipoproteins as substrates for lipid transfer *in vitro* (Ando *et al.*, 1990; Ryan *et al.*, 1990d; Silver *et al.*, 1990; Singh and Ryan, 1991; Singh *et al.*, 1992b). Human LDL, which does not contain DAG, serves as an excellent acceptor of DAG from HDLp. It has proven very useful in lipid transfer reactions since the donor HDLp and acceptor LDL can be easily separated by density gradient ultracentrifugation when necessary (Ryan *et al.*, 1990d). LTP-facilitated DAG net transfer from HDLp to LDL resulted in a stable DAG-depleted, very high density lipophorin and a lower density LDL without the transfer or exchange of insect or human apolipoproteins. Cholesterol ester (CE), free cholesterol, and PL net transfer also occurred but to a lesser extent than DAG. LDL was also shown to act as a donor of DAG when [^3H]DAG-LDL was incubated with HDLp and LTP-facilitated transfer of [^3H]DAG from LDL to HDLp was observed. DAG transfer, therefore, was bidirectional but net transfer was from HDLp to LDL (Ryan *et al.*, 1990d). The stable very-high-density lipophorin particle produced by DAG transfer to LDL by LTP may represent the basic matrix structure of lipophorin described above (Ryan, 1990). It is similar in density and lipid content to VHDLp-E (Kawooya and Law, 1988a) and lipophorin isolated from the hemolymph of animals reared on a fat-free

diet (Prasad *et al.*, 1986b). The transfer of DAG out of lipophorin particles may require the partitioning of DAG between the core and the surface of the particle and the movement of DAG from the core to the surface as DAG is removed to produce the lipid-depleted lipophorin particles observed. Structural studies of lipophorin described above support the concept that DAG can be found both in the core and at the surface of the lipophorin particle.

LTP has also been shown to catalyze a disproportionation reaction similar to that of LDLp with human high density lipoprotein (HDL) (Silver *et al.*, 1990). When HDL was incubated with catalytic amounts of LTP, a rearrangement of lipid and apoproteins occurred producing a low density lipoprotein particle and free apolipoprotein A-1 (apo A-1). The low density lipoprotein product contained apolipoprotein A-2 (apo A-2) as its major lipoprotein and the lipid originally associated with HDL was recovered with this fraction in the same ratio as in the substrate. These results suggest that LTP-induced lipid transfer altered the distribution of lipid between HDL particles such that apoprotein migration and particle disintegration occurred. The lower surface area to core volume ratio of the larger, less dense particles required less surface material, thus, allowing apoprotein dissociation. Apo A-1 may have dissociated from the particles preferentially to apo A-2 due to its lower affinity (Silver *et al.*, 1990). The similarity in the reactions of LTP with insect LDLp and human HDL, in which apoprotein migration occurs, seems to be rooted in the presence of exchangeable apoproteins. Particle disintegration may not have occurred in the LDLp case due to the presence of the integral apoLp-I and apoLp-II. Similar reactions have been observed for the mammalian plasma cholesteryl ester transfer protein (CETP) and phospholipid transfer protein (PLTP) indicating analogies in the transfer abilities of these

transfer proteins with LTP (Barter *et al.*, 1988; Jauhiainen *et al.*, 1993; Tu *et al.*, 1993).

The transformation of HDL by LTP was exploited in a method for the non-denaturing isolation of human apo A-1 from HDL (Ryan *et al.*, 1992b). Previously, HDL apolipoproteins were liberated by organic extraction or exposure to a chaotropic salt. Apo A-1 isolated by the action of LTP described above was similar to apo A-1 purified by organic extraction in its tendency to self-associate, α -helical content, and activation of lecithin : cholesterol acyltransferase (Ryan *et al.*, 1992b).

LTP is capable of facilitating the transfer of both polar and apolar lipids. The relative rates of LTP-facilitated transfer of DAG and hydrocarbon were examined in two different systems. Singh and Ryan (1991) radiolabelled the lipid components of *M. sexta* HDLp-A by injection of [^{14}C]acetate into moths and incubated radiolabelled HDLp-A with LDL and catalytic amounts of *M. sexta* LTP. Analysis of the lipids recovered in acceptor LDL by thin layer chromatography and radiochromatogram scanning revealed that LTP preferentially transferred DAG in the initial stages of the reaction. Hydrocarbon transfer occurred at a slower rate in the initial stages and then at a greater rate than DAG in later stages. HDLp-A retained a small portion of its DAG component while hydrocarbon transfer proceeded to completion suggesting this DAG is either essential in maintaining lipophorin structural integrity (Singh and Ryan, 1991) or is inaccessible to LTP. The initial preference of LTP-induced transfer for DAG above hydrocarbon in this study may reflect either a preference of LTP for DAG or the relative availability or absolute amount of DAG over hydrocarbon in the lipophorin particle.

When the transfer ability of *P. americana* LTP was examined by a membrane-bound donor lipophorin assay, hydrocarbon was found to transfer at a greater rate than DAG between *P. americana* HDLp particles. DAG and hydrocarbon were both transferred from *P. americana* HDLp to *L. migratoria* LDLp by *P. americana* LTP and hydrocarbon was transferred from *L. migratoria* LDLp to *P. americana* HDLp (Takeuchi and Chino, 1993). The rate of transfer of DAG from *L. migratoria* LDLp to *P. americana* HDLp would have been useful to examine the effects of lipophorin lipid composition on relative rates of lipid transfer by LTP. *P. americana* HDLp was a higher hydrocarbon and lower DAG content than *M. sexta* HDLp. The differences in the relative lipid compositions of *M. sexta* versus *P. americana* lipophorins may be the determining factor in the rate of transfer of diacylglycerol versus hydrocarbon from these lipophorins.

The lipid substrate specificity of LTP was further examined in the transfer of radiolabelled lipids from HDLp to LDL (Singh *et al.*, 1992b). Unesterified cholesterol spontaneously transfers between lipoproteins while LTP facilitates the transfer of CE and phosphatidylcholine, the latter at a faster rate. Phosphatidylcholine, however, seems to be subject to LTP-induced exchange between lipoproteins rather than net transfer. The origin of the difference in transfer rate of phosphatidylcholine and CE is unknown but may be an inherent preference of LTP for polar lipids or the relative availability of phosphatidylcholine versus CE in the lipophorin particle. When the capacity of LDL to receive exogenous lipid from lipophorin was tested, HDLp : LDL protein ratios of greater than 3 : 1 resulted in sample turbidity which was attributed to the breakdown and aggregation of the LDL particles as demonstrated by electron microscopy (Singh *et al.*, 1992b). This observation indicated LDL has a finite capacity to accept exogenous lipid. Sample turbidity

was prevented and lipid content and overall particle size of lipid-loaded LDL particles increased when exchangeable apolipoproteins such as apoLp-III and human non-apoB VLDL apoproteins were included in the transfer reaction. This indicates that additional surface components are required for LDL to maintain structural stability when loaded beyond its inherent capacity for lipid (Singh *et al.*, 1992b).

An artificial system of triolein/PL microemulsions stabilized by the human exchangeable apolipoprotein, apo A-1, and human LDL were used as donor and acceptor lipid particles. This system demonstrated that LTP can facilitate net transfer of TAG and PL without exchange or transfer of apoproteins or reciprocal CE transfer from LDL to microemulsion (Ando *et al.*, 1990). The rate of PL transfer was twice that of TAG transfer. This activity is in contrast to CETP activity in the same system where CETP catalyzed the reciprocal transfer of CE from LDL to microemulsion and TAG from microemulsion to LDL until a homogeneous distribution of TAG and CE was achieved (Nishikawa *et al.*, 1988).

The mode of lipid rearrangement and the final equilibrium distribution of lipids catalyzed by LTP seems to depend upon the nature of the substrate donor/acceptor lipid particle pair rather than an inherent characteristic of LTP. LTP catalyzes exchange of DAG between unrelated insect lipoproteins, *M. sexta* HDLp and *H. zea* very-high-density chromolipoprotein. DAG transfer as well as exchange, however, occurs from LDLp to HDLp-L to produce an intermediate lipophorin subspecies (Ryan *et al.*, 1986a; Ryan *et al.*, 1988c), from donor LDLp to acceptor LDLp particles to produce a VLDLp and a HDLp (Ryan *et al.*, 1990c), and from HDLp-L to LDL resulting in a very high density lipophorin and a lower density LDL (Ryan *et al.*, 1990d). LTP also catalyzes TAG transfer from a

triolein/PL microemulsion to LDL (Ando *et al.*, 1990). The proportional amounts of substrate particle DAG or TAG may play a role in the determination of the direction of net transfer. For example, LDL, which lacks DAG, seems to act as a sink of DAG transferred from insect lipophorins (Ryan *et al.*, 1990d; Singh *et al.*, 1992b). Studies with LTP isolated from *P. americana* demonstrated that hydrocarbon is preferentially transferred from *P. americana* lipophorin above DAG. This appears to be dependent upon the relative amounts of hydrocarbon and DAG found in the particular lipophorin species (Takeuchi and Chino, 1993). Transfer between insect lipophorins seems to occur in the direction of lipid-rich donor to relatively lipid-poor acceptor to produce an intermediate species. Transfer between HDLp and LDL, however, occurs from the relatively lipid-poor, DAG-rich HDLp to the lipid-rich but DAG-poor LDL. These two systems differ in that all lipophorins possess an identical basic matrix structure while HDLp and LDL have distinct structures. LDL may provide a hydrophobic environment which is better equipped to sequester DAG than lipophorin (Ryan *et al.*, 1990d). Similarly, LTP facilitates the transfer of PL and TAG from microemulsion particles to LDL supporting the hypothesis that LDL provides a friendlier environment for lipids (Ando *et al.*, 1990).

1.9 LTP transfer activity assays.

A number of assays of LTP activity based on the ability of LTP to transfer lipid between lipoproteins have been developed. LTP was initially isolated with the use of a lipid transfer assay which followed transfer activity through the purification scheme. This assay was labour intensive and monitored the

shift in density of substrate lipophorins to a single intermediate subspecies after incubation in the presence of LTP (Ryan *et al.*, 1986b). Donor lipophorin (HDLp-L), radiolabelled in its DAG moiety, has also been used in LTP activity assays. In this case, LTP-facilitated DAG exchange activity was measured by incubation of LDLp with radiolabelled HDLp-L subsequently separated by density gradient ultracentrifugation (Ryan *et al.*, 1988c). Takeuchi and Chino (1993) developed an assay of transfer activity for *P. americana* LTP which involved the binding of HDLp, radiolabelled in its lipid moiety, to PVDF membrane and incubation of this membrane with acceptor lipophorin in the presence and absence of LTP. These assays do not allow the quantitation of facilitated exchange since it is difficult to reisolate donor and acceptor lipoproteins after lipid transfer due to the equilibration of densities of the participating lipoproteins. Assays utilizing structurally differing lipoproteins were, therefore, developed. The blue, very-high density, biliverdin-containing, chromolipoprotein from *H. zea* proved useful in quantitating facilitated exchange since net transfer between *H. zea* VHDL and lipophorin does not occur. The rate of LTP-induced DAG exchange was found to be 2.5 $\mu\text{g DAG/min}/\mu\text{g protein}$ at 25°C. The linearity of the LTP-facilitated DAG exchange up to 0.3 $\mu\text{g LTP}$ made it possible to determine the amount of LTP in *M. sexta* hemolymph throughout development (Ryan *et al.*, 1988b).

The ability of LTP to transfer [^3H]DAG from HDLp-L to human LDL was also exploited in an assay of LTP-facilitated net lipid transfer (Ryan *et al.*, 1990d). It is possible to measure transfer with this system because LDL is void of DAG. Any DAG recovered with LDL is the result of transfer. The ease with which LDL is separated from HDLp made this a convenient assay. LTP-catalyzed transfer of DAG is linear as a function of LTP concentration and time

when a 1 : 2 HDLp-L : LDL protein ratio is used. The rate of transfer in the initial phase of the reaction is 2.4 $\mu\text{g DAG/min}/\mu\text{g LTP protein}$ (Ryan *et al.*, 1990d).

Recently, a novel assay of LTP activity was developed based on the ability of LTP to induce the structural breakdown of LDL particles upon the transfer of large amounts of DAG from HDLp-W1 (Singh *et al.*, 1992a,b). When LTP is incubated with LDL and HDLp-W1 in protein ratios greater than 3 : 1 HDLp-W1 : LDL, a temperature, time, LTP, LDL, and HDLp-W1 concentration dependent increase in sample turbidity, quantifiable by determination of the absorbance, occurs. This assay is significantly more convenient than those previously developed since no radioactive substrates are required, the substrates are easily obtained, the separation of product lipoproteins is not necessary, and the reactions can be carried out and quantitated in a microtiter plate (Singh *et al.*, 1992a).

Prior to the work presented in this dissertation, little was known about the mechanism of action of LTP or the importance of the apoprotein and lipid moieties in transfer activity. The role of the DAG component of LTP was examined by Ryan *et al.* (1988c) in studies where HDLp-L, radiolabelled in its DAG component, was incubated with substrate quantities of LTP. Upon separation of the lipoproteins by density gradient ultracentrifugation, fractionation, and radiolabel quantitation, one quarter of the recovered radioactivity was found to be associated with fractions corresponding to the density of LTP. Incubation of [^3H]DAG-LTP with unlabelled HDLp-L resulted in the transfer of [^3H]DAG from LTP to HDLp-L. These results demonstrated that the DAG component of LTP is in dynamic equilibrium with that of substrate lipophorin and has more than a merely structural role in the LTP

complex (Ryan *et al.*, 1988c). Additional evidence for the role of DAG in lipid transfer was obtained when lipid extraction of LTP resulted in a loss of lipid transfer activity (Ryan *et al.*, 1988c). The ability of LTP apoproteins to catalyze DAG transfer in the absence of DAG was also examined when LTP apoproteins were bound to nitrocellulose after SDS-PAGE and incubated in a DAG transfer assay. LTP apoproteins were not capable of inducing DAG transfer under these conditions (Ryan *et al.*, 1988c). These experiments indicate that the DAG component of LTP has an important role in the lipid transfer activity of the complex, however, the denaturation of LTP must be considered in the interpretation of some of these results.

1.10 Thesis objectives.

The complexity of LTP with its multimeric structure including five or six polypeptides, the presence of lipid, unique morphology, and catalytic activity poses many questions regarding structure and function relationships. The work described in this thesis examines a number of these problems. Questions that will be addressed are: 1) does LTP catalyze lipid transfer between lipoproteins via a carrier-mediated mechanism or is a ternary complex of LTP with its substrate lipoproteins required; 2) are the apoproteins of LTP related; 3) what is/are the catalytically important apoprotein(s); 4) are any of the apoproteins, particularly apoLTP-III, buried within the particle; 5) how stable is the complex; and 6) what is the nature of the interaction of apoLTP-III with the rest of the particle?

Figure 1.1 Model of lipid mobilization and transport by lipophorin.

Mobilization of fat body lipid stores is signalled by the release of adipokinetic hormone (AKH) from the corpora cardiaca. Binding of AKH to an adenylate cyclase-coupled receptor results in the conversion of ATP to cAMP. cAMP activates a hormone-sensitive lipase which converts triacylglycerol (TAG) to diacylglycerol (DAG). DAG is loaded into lipophorin particles (lipophorin loading intermediates, Lp-I) at the surface of the fat body through the action of lipid transfer particle (LTP). Concomitant with DAG loading, additional apolipophorin-III (apoLp-III) molecules associate with the lipophorin intermediate to produce the lipid-loaded low density lipophorin (LDLp). LDLp transports DAG to the flight muscle where hydrolysis by a lipophorin lipase (LpL) produces free fatty acid (FFA) which is taken up by the flight muscle. As DAG is removed from LDLp, apoLp-III molecules dissociate to produce high density lipophorin (HDLp-A) which returns to the fat body to be reloaded with DAG. Modified from Ryan (1990).

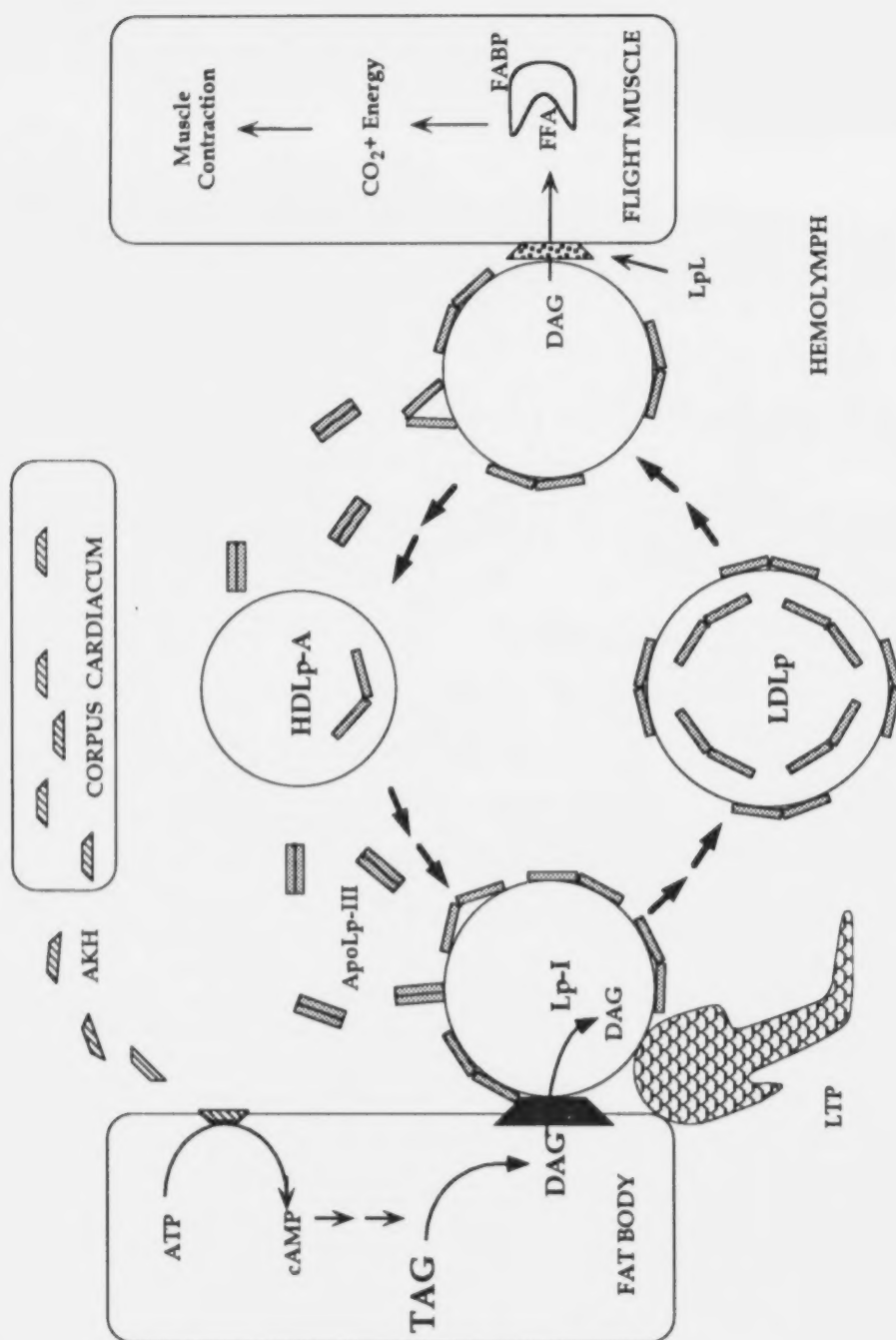


Figure 1.2 SDS-PAGE of lipid transfer particle.

A 4 - 12 % linear acrylamide gradient SDS-PAGE gel loaded with 50 μ g LTP was electrophoresed at 30 mA constant current for 4 h (lane 2). Molecular weight markers (lane 1) including myosin, 200 000; β -galactosidase, 116 000; phosphorylase *b*, 93 000; albumin, 67 000, and ovalbumin, 45 000 are shown for comparison. Molecular weights for LTP apoproteins were estimated to be apoLTP-I, ~320 000; apoLTP-II, 85 000; and apoLTP-III, 55 000.

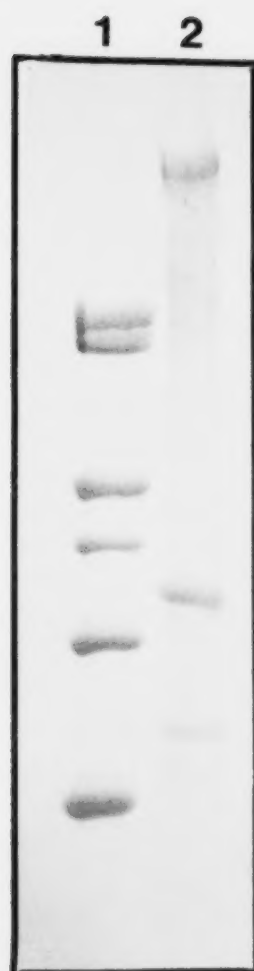


Table 1.1 Properties of *M. sexta* (larval), *L. migratoria* (adult), and *P. americana* (adult) hemolymph lipid transfer particle.

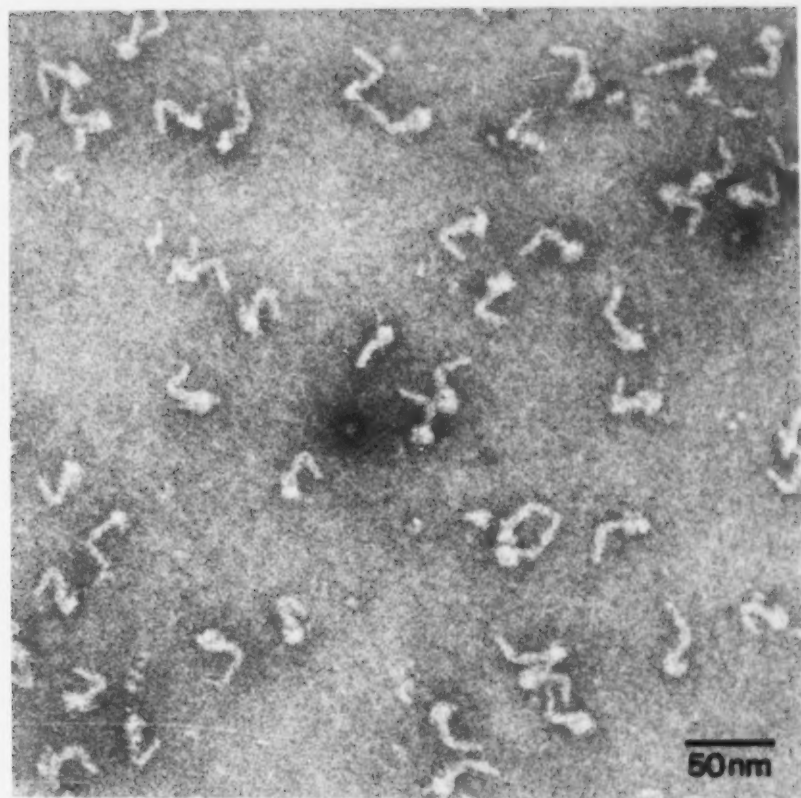
	<i>M. sexta</i>	<i>L. migratoria</i>	<i>P. americana</i>
<u>Apoproteins</u>	M _r	M _r	M _r
apoLTP-I	~320 000	310 000	310 000
apoLTP-II	85 000	89 000	94 000
apoLTP-III	55 000	68 000	61 000
<u>Lipid</u>	% weight	% weight	% weight
Phospholipid	58	18	57
Diacylglycerol	28	44	2
Free Fatty Acid	8	13	nd
Triacylglycerol	6	nd	nd
Hydrocarbon	nd	17	40
Cholesterol	nd	8	trace

nd = not determined

references: *M. sexta* (Ryan *et al.*, 1988c), *L. migratoria* (Hirayama and Chino, 1990), *P. americana* (Takeuchi and Chino, 1993).

Figure 1.3 Electron micrograph of lipid transfer particle.

Electron micrograph of *M. sexta* LTP negatively stained with 2 % sodium phosphotungstate.



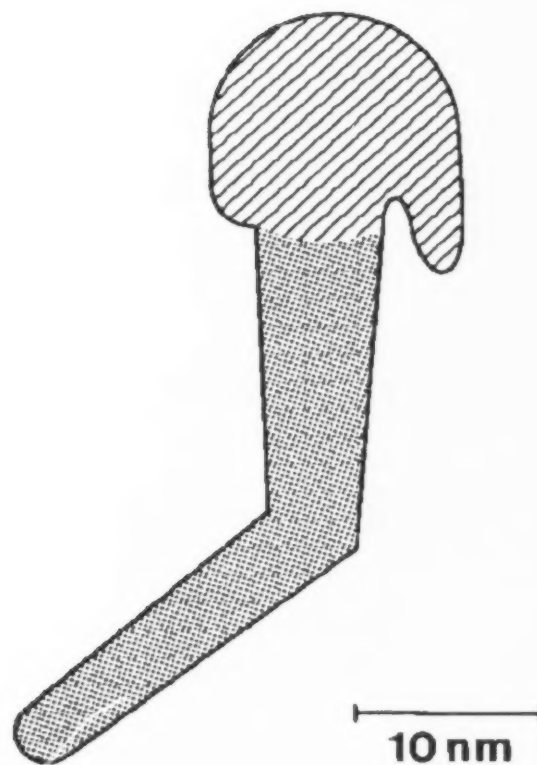


Figure 1.4 Model of lipid transfer particle.

Representational drawing of *M. sexta* LTP based on electron micrographs. Reproduced from Ryan *et al.*, 1990b.

CHAPTER 2

MATERIALS AND METHODS

portions of this chapter were previously published:

Blacklock, B. J., Smillie, M., and Ryan, R. O. (1992) Insect lipid transfer particle can facilitate net vectorial lipid transfer via a carrier-mediated mechanism. *J. Biol. Chem.* **267**, 14033-14037.

2.1 Materials.

Materials were obtained from the following sources: ECL chemiluminescent detection system, [9, 10-³H]oleic acid, and Na¹²⁵I; Amersham Corp., agar, ascorbic acid, and Tris-HCl; Anachemia, boric acid, EDTA, glycerol, glycine, KBr, NaCl, CH₃COONa, MgCl₂, NaHCO₃, Na₂HPO₄, NaH₂PO₄, and Tween-20; BDH, acrylamide, bis-acrylamide, Biogel P-6DG desalting gel, diethylaminoethyl (DEAE) Biogel A anion exchange gel, gelatin, and SDS-PAGE molecular weight standards, high range; Biorad Laboratories, 2,2'azino-di-[3-ethylbenzthiazoline] sulfonate (ABTS) substrate, anti human apolipoprotein-B, trypsin, and soybean trypsin inhibitor; Boehringer Mannheim Biochemica, biotinylated goat anti rabbit IgG, streptavidin-horse radish peroxidase, and Tris-base; Gibco BRL, diet vitamin mixture; Hoffman-La Roche, Ecolite, casein, salt mix W, and sodium dodecyl sulfate; ICN Biomedicals, KCl, β-mercaptoethanol, and NaN₃; J. T. Baker Inc., sorbic acid; Kodak, bicinchoninic acid protein assay system, horse-radish peroxidase-conjugated goat anti-rabbit IgG, and Iodogen beads; Pierce Chemical Co., activated thiol-Sepharose 4B, Sephadex G-150 gel filtration gel, Sepharose CL 4B, and Sepharose CL 6B; Pharmacia LKB Biotechnologies, Ribi's adjuvant system; Ribi ImmunoChem Research Inc. Cedar Lane Laboratories, dithiothreitol (DTT), and guanidine-HCl; Schwarz/Mann Biotech, bovine serum albumin (fraction V), 3-[cyclohexylamino]-1-propanesulfonic acid (CAPS), Coomassie Blue R-250, CNBr-activated Sepharose 4B, diisopropylfluorophosphate (DFP), formaldehyde, Freund's adjuvant system, glutathione, Nonidet P-40 (NP-40), phenylmethylsulfonyl fluoride (PMSF), Protein-A agarose, Protein-A Sepharose, and urea; Sigma, and wheat germ,

methyl paraben, ampicillin trihydrate, and kanamycin sulfate; United States Biochemical.

2.2 Animals and diet.

All life stages of *M. sexta* utilized were obtained from a continuous colony reared in the laboratory on an artificial diet consisting of wheat germ, casein, salts, methyl paraben, sorbic acid, ascorbic acid, other vitamins, and antibiotics and formaldehyde to prevent spoilage in an agar base (Prasad *et al.*, 1986b). Larval *M. sexta* were housed individually in small plastic cups at 22°C until the fifth instar, prepupal stage (approximately 1 month) when they were transferred to wooden blocks for pupation (approximately 2 weeks). Upon eclosion, adult moths were housed in a well-ventilated cage under 16 h light/8 h dark conditions and allowed to mate and oviposit. Eggs were collected from tobacco or tomato plants and hatched at 22°C. Hemolymph was collected at larval and prepupal life stages.

Adult female New Zealand white rabbits were used for antibody production.

2.3 Lipid transfer particle and lipoprotein isolation.

Purification of LTP, lipophorin, and LDL took advantage of the buoyant properties of these lipoproteins. LTP was isolated according to a procedure modified from Ryan *et al.* (1990b) from hemolymph of 7 day-old, fifth instar, prepupal *M. sexta* larvae. Animals were anesthetized by chilling on ice and

bled through incisions above both second prolegs into 40 ml bleeding buffer for 80 animals (bleeding buffer: phosphate buffered saline (PBS), 0.1 M sodium phosphate/0.15 M NaCl/5 mM EDTA pH 7.0 plus 0.2 mM PMSF/1.0 mM DFP/2.0 mM glutathione). Hemolymph was centrifuged in a JA 20 rotor (Beckman) in a J2-21 centrifuge (Beckman) at 10 000 rpm for 15 min at 4°C then filtered through cheesecloth, KBr was dissolved in the hemolymph (8.9 g per 20 ml final volume) and quick-seal ultracentrifuge tubes (39 ml; Beckman) were half-filled with 20 ml hemolymph, over layered with density = 1.04 g/ml solution (0.93 M KBr/0.15 M NaCl/0.02 % NaN₃), and ultracentrifuged at 50 000 rpm for 20 h at 4°C in a Beckman VTi50 rotor, L8-55 Beckman ultracentrifuge. LTP-containing bands were removed from the tubes with a syringe and needle, concentrated by Omegacell stir cell (100K cutoff, Amersham Corp.) to 15 ml, applied to 500 ml Sepharose CL 6B column, and eluted in 7 ml fractions with 50 mM NaH₂PO₄/0.02 % NaN₃ pH 7.0. Absorbance (280 nm) of fractions was determined and those containing LTP were applied to a DEAE ion exchange column. The column was washed with 50 mM NaH₂PO₄/0.02 % NaN₃ pH 7.0 and LTP was eluted with 50 mM NaH₂PO₄/300 mM NaCl/0.02 % NaN₃ pH 7.0. A second density gradient ultracentrifugation step was employed to further purify and concentrate LTP. KBr (8.9 g per 20 ml final volume) was dissolved in the LTP-containing fractions from the ion exchange column, quick seal tubes were half filled, over layered with density = 1.21 g/ml solution (2.5 M KBr/0.15 M NaCl/0.02 % NaN₃) and subjected to density gradient ultracentrifugation (VTi50 rotor, 50 000 rpm, 18 h, 4°C). LTP was collected from the top of the tubes and concentrated by stir cell.

[³H]DAG-labelled HDLp-L was isolated from fasted (4 h) 4 day-old fifth instar larvae. [^{9,10-³H}]Oleic acid (10 Ci/mmol; 10 µL per animal, dried down

under N₂, and brought up to volume in ethanol) was administered by bolus, animals were bled after 30 min (as described above), and collected hemolymph was subjected to density gradient ultracentrifugation as previously described (Ryan *et al.*, 1988c; Ryan *et al.*, 1990c). Hemolymph was prepared for ultracentrifugation by dissolving 8.9 g KBr in 20 mL centrifuged and filtered hemolymph. Quick seal tubes were half-filled and over layered with 0.15 M NaCl/0.02 % NaN₃. Ultracentrifugation was conducted in a Beckman Ti50.2 rotor at 50 000 rpm at 4°C for 16 h. HDLp-L was collected from the middle of the tube, dialyzed into PBS, density adjusted by addition of 6 g KBr per 20 ml final volume, over layered with density = 1.21 g/ml solution (2.5 M KBr/0.02 % NaN₃), and ultracentrifuged at 40 000 rpm for 20 h at 4°C in a Ti70.1 rotor (Beckman). HDLp-L was then collected from the top of the tube. Unlabelled HDLp-L was isolated in a similar manner. HDLp-W1 was collected from the top of the tube after the initial density gradient ultracentrifugation of the LTP isolation procedure. Arylphorin was previously isolated from *M. sexta* larvae as described (Ryan *et al.*, 1985a).

Human LDL was isolated from freshly collected plasma between the density limits of 1.006 and 1.063 g/ml by sequential ultracentrifugation. Plasma was ultracentrifuged in 26 ml polycarbonate tubes (Beckman) at 40 000 rpm in a Ti50.2 rotor for 20 h at 4°C, VLDL and chylomicrons were collected from the top of the tubes after ultracentrifugation. The remainder of the tubes were density adjusted to 1.063 g/ml by the addition of KBr and ultracentrifuged for 20 h at 4°C at 40 000 rpm in a Ti50.2 rotor. LDL was collected from the top of tubes and further purified by an additional ultracentrifugation step at density = 1.063 g/ml.

Protein concentrations were determined by the bicinchoninic acid assay method using bovine serum albumin as a standard.

2.4 Radioiodination of LDL and LTP.

LDL and LTP were radioiodinated with Iodogen beads using Na¹²⁵I as described by Markwell (1982). Briefly, LDL (0.32 mg) or LTP (0.5 mg) was dialyzed into PBS overnight and incubated with 2 Iodo-beads pre-loaded with 0.1 mCi Na¹²⁵I for LDL or 0.5 mCi for LTP, for 20 min at 22°C. Excess free ¹²⁵I was removed by gel filtration with a 10 ml BioGel P-6DG desalting column with PBS and fractions containing radiolabelled protein were pooled. The specific activity of ¹²⁵I-apolipoprotein B-LDL was 1.67×10^5 cpm/ μ g.

2.5 Binding of lipoproteins to Sepharose matrices.

Human LDL and [³H]DAG-HDLp-L were bound to CNBr-activated Sepharose 4B and activated thiol-Sepharose 4B, respectively, according to the manufacturer's instructions. LDL (5 mg LDL protein/ml hydrated gel) was dialyzed against 0.1 M borate/0.5 M NaCl pH 8.5 prior to coupling with the CNBr-activated Sepharose 4B. After coupling, excess groups were blocked by incubation with 0.5 M Tris-HCl/0.5 M NaCl pH 8.0. Non-specifically bound protein was removed by alternate washings with the coupling buffer and 0.5 M CH₃COONa/0.5 M NaCl pH 4.0. Typical CNBr-activated Sepharose 4B binding efficiencies for LDL bound by this method are 96% (Morton, 1985). [³H]DAG-HDLp-L (3 mg protein/ml hydrated gel) was dialyzed against 0.1 M Tris-HCl/0.5 M NaCl/1 mM EDTA pH 7.5 prior to coupling to activated thiol-

Sepharose 4B. Coupling was accomplished by incubation of ligand with hydrated gel at room temperature for 1-2 hours with gentle end-over-end shaking. Typical binding efficiency for [^{3} H]DAG-HDLp-L was 91 % as determined by recovery of radioactivity in [^{3} H]DAG-HDLp-L-thiol-Sepharose 4B. When necessary, the lipoprotein-Sepharose was diluted with Sepharose CL 4B. In some experiments [^{3} H]DAG-HDLp-L was eluted from the Sepharose beads with 50 mM DTT/0.1 M Tris-HCl/0.5 M NaCl/1 mM EDTA pH 7.5.

2.6 Circulation system and transfer assay.

All mechanism circulation experiments were carried out with the same system which allowed the circulation of PBS between two columns (Figure 2.1). Columns (Pharmacia C-10 with AC-10 adapters) packed to a 2 ml bed volume of LDL-CNBr-activated Sepharose 4B, [^{3} H]DAG-HDLp-L-thiol-Sepharose 4B, or Sepharose CL 4B were connected in series with a peristaltic pump and an open reservoir permitting additions to the system. The solution in the reservoir was gently stirred throughout the circulation. The volume of the system was 8 ml circulated at a rate of 0.54 ml/min.

The dependence of [^{3} H]DAG transfer from HDLp-L-thiol Sepharose to LDL-CNBr-Sepharose on LTP concentration was determined in preliminary experiments. [^{3} H]DAG-HDLp-L-thiol Sepharose (2.5 mg [^{3} H]DAG-HDLp-L protein; specific activity = 1.1×10^5 cpm/mg protein) and LDL-CNBr-Sepharose (5 mg LDL protein) were packed into separate columns and LTP was circulated at concentrations ranging from 0 to 100 μ g/ml for 16 hours at 23°C. Transfer of [^{3} H]DAG to LDL-CNBr-activated-Sepharose was measured by washing the column with PBS, rinsing the matrix into a 20 ml glass scintillation vial,

removing the supernatant PBS, adding 10 ml scintillation fluid (Ecolite) and counting 24 hours after vigorous shaking (20 sec). This provided an efficient method of detecting transferred radioactivity since the lipid was extracted from the solid-phase LDL by the Ecolite.

Further experiments were carried out with [^3H]DAG-HDLp-L-thiol Sepharose (0.6 mg [^3H]DAG-HDLp-L protein; specific activity = 4.4×10^5 cpm/mg protein) or unbound [^3H]DAG-HDLp-L (equivalent [^3H]DAG-HDLp-L concentration and specific activity) and LDL-CNBr-Sepharose (1.0 mg LDL protein) or unbound LDL (1.0 mg protein). When both lipoproteins were bound, LTP (25 $\mu\text{g}/\text{ml}$) or an unrelated storage protein, arylphorin (25 $\mu\text{g}/\text{ml}$), were circulated for 16 hours at 23°C. When only [^3H]DAG-HDLp-L or only LDL were bound, LTP (25 $\mu\text{g}/\text{ml}$) was circulated for 4 hours at 23°C and when both lipoproteins were unbound, 6.3 $\mu\text{g}/\text{ml}$ LTP was circulated for 1 hour at 23°C. Circulated LDL was collected by draining and washing the columns connected in series with a total of 15 ml PBS. KBr (3 g) was dissolved in the sample and the volume was brought up to 20 ml with PBS. The samples were placed in 39 ml quick seal tubes, over layered with 0.15 M NaCl and ultracentrifuged for 16 hours at 50 000 rpm at 4°C in a VTi-50 rotor. After ultracentrifugation the tubes were fractionated into 1.1 ml fractions and transfer of [^3H]DAG to unbound LDL was determined by liquid scintillation counting of 50 μl aliquots of each fraction. The density of each fraction was determined by refractometry.

Calculation of the rate of DAG transfer was based on the original specific activity of [^3H]DAG-HDLp-L and the radioactivity recovered in LDL after lipid transfer. Spontaneous transfer rates were determined from incubations lacking LTP. Facilitated transfer rates were determined by correcting LTP-containing

assays for spontaneous transfer. In all cases, spontaneous transfer was less than 8 % of the values obtained for the corresponding LTP containing incubation. In all transfer experiments the total counts recovered in LDL was less than 50 % of the original counts in [^3H]DAG-HDLp-L indicating that the rates of transfer were within the linear range of the LTP activity response (Ryan *et al.*, 1990d).

2.7 Lipid transfer assay.

LTP-facilitated net transfer of DAG from HDLp-W1 to LDL, when HDLp-W1 is present in greater than 3-fold excess, results in a breakdown of the basic matrix structure of LDL particles and aggregation. Sample turbidity resulting from LDL particle breakdown and aggregation is dependent upon LTP and substrate lipoprotein concentration, time, and temperature and can be quantitated by measuring the absorbance at 340 nm (Singh *et al.*, 1992a,b). LTP activity was assayed by incubation with 500 μg HDLp-W1 protein and 100 μg LDL protein in a total volume of 100 μl in a microtiter plate at 37°C for 30 min or as indicated. Turbidity development was measured by determining the absorbance at 340 nm on a microtiter plate reader (EAR 340AT Easy Reader microtiter plate reader, SLT-Labinstruments, Austria).

2.8 Preparation of polyclonal antibodies.

Polyclonal anti sera were raised against apoLTP-I, apoLTP-II, and apoLTP-III by repeated intramuscular injection of isolated apoproteins into rabbits. Apoproteins were isolated by SDS-PAGE (4 - 15 % linear acrylamide gradient) of LTP (1 mg) by the method of Laemmli (Laemmli, 1970) using a

preparative (3 mm) gel. In the case of apoLTP-I and apoLTP-III, gel slices were excised from the Coomassie Blue stained gel, lyophilized, macerated, and injected into rabbits with Freund's adjuvant system. This procedure did not produce antibodies against apoLTP-II even after repeated attempts with 3 rabbits. Anti apoLTP-II serum was obtained by injection of apoLTP-II, eluted from macerated gel slice by end-over-end shaking with elution buffer (50 mM Tris-HCl/0.15 M NaCl/0.1 % SDS pH 8.0) for 48 h, into a rabbit with Ribi's adjuvant. Non-immune serum was obtained by collecting blood from rabbits prior to injection of antigen.

Polyclonal antibodies against native LTP were produced by repeated injection of purified LTP (1 mg) into a rabbit with Freund's adjuvant. Anti larval serum protein serum prepared previously (Ryan *et al.*, 1985b) was used as an irrelevant antibody.

2.9 Immunoblotting of purified LTP.

Purified LTP (0.2 μ g) was probed with anti and non-immune sera after transfer from a 4 - 15 % linear acrylamide gradient non-reducing SDS-PAGE gel (Laemmli, 1970) to PVDF membrane (Immobilon, Millipore Corp.) under transfer conditions suitable for high molecular weight proteins (50 mM Tris-base/380 mM glycine pH 8.3; 500 mAmps, 3 h, 4°C with cooling coil). Reducing conditions in SDS-PAGE are not required since apoproteins of purified LTP are not covalently linked by disulfide bridges (Ryan *et al.*, 1988b). The membrane was blocked by gentle shaking for 30 min with 20 mM Tris-base/150 mM NaCl/0.2 % Tween-20 pH 7.2 (TTBS) then incubated with non-immune serum (1 : 2 500 dilution made in TTBS), anti holoLTP (1 : 15 000), anti

apoLTP-I (1 : 10 000), anti apoLTP-II (1 : 5 000), or anti apo-III (1 : 2 500) for 60 min with gentle shaking. The membranes were then incubated (30 min) with biotinylated goat anti-rabbit IgG (1 : 5 000 in TTBS) followed by streptavidin-horse radish peroxidase (30 min, 1 : 5 000 in TTBS). Extensive washing with TTBS followed each incubation. Detection was achieved by incubation with luminol, a chemiluminescent horse-radish peroxidase substrate (ECL) and autoradiography.

2.10 Purification of immunoglobulin G.

Immunoglobulin G (IgG) were purified from anti and non-immune sera with Protein-A agarose as described by Harlow and Lane (1988). Briefly, the pH of the sera was adjusted to pH 8 by the addition of 1/10 volume 1.0 M Tris-base pH 8.0. The sera were applied to Protein-A agarose columns and washed with 10 times column volume each 100 mM Tris-base pH 8.0 and 10 mM Tris-base pH 8.0. IgG molecules were eluted with 100 mM glycine pH 3.0, concentrated, and dialyzed into PBS.

2.11 Characterization of apoprotein-specific antibodies.

Antibody capture enzyme-linked immunosorbent assay (ELISA) were used to characterize the binding of the apoprotein-specific antibodies to native LTP. The procedure used was modified from that described by Harlow and Lane (1988). Briefly, LTP (1 µg) was bound to the well of Immulon 3 microtiter plates (Dynatech Laboratories) in coating buffer (0.5 M NaHCO₃/0.1 % NaN₃ pH 9.6) and excess protein binding sites were blocked with 0.5 % gelatin/PBS

(blocking agent). Apoprotein-specific and irrelevant antibodies were diluted in blocking agent to equivalent IgG concentrations as indicated. Triplicate wells for each antibody dilution (200 μ l per well) were prepared and incubated 22°C for 2 h. Following extensive washing, horse-radish peroxidase conjugated goat anti rabbit IgG diluted in blocking agent (1 : 3 000) was added and incubated at 22°C for 2 h. ABTS substrate solution was added to the wells after washing and the absorbance at 405 nm measured after 6 min incubation at room temperature. Half maximal binding values were interpolated from each IgG concentration curve as the IgG concentration at which 50 % of the maximal change in absorbance (405 nm) was observed.

Antigen competition antibody capture ELISA experiments were carried out essentially as above except only one IgG concentration was used; the half maximal binding value, and either 100-fold excess LTP or buffer were added to the wells prior to the addition of primary antibody.

The antibody capture ELISA experiment was also carried out with 0.05 % Tween-20 included in the blocking agent and primary and secondary antibody incubations.

2.12 Effect of LTP apoprotein-specific IgG on lipid transfer activity.

Apoprotein-specific antibodies were used to examine the role of individual LTP apoproteins in lipid transfer. The lipid transfer assay described above was used to determine the lipid transfer activity of immune complexes of LTP and apoprotein-specific IgG. LTP (0.75 μ g; within the linear range of LTP

turbidimetric response) was incubated with varying amounts of apoprotein-specific or non-immune IgG in a total volume of 80 μ l for 16 h at 4°C. LDL (100 μ g protein) and HDLp-W1 (500 μ g protein) were then added to the immune complexes, incubated at 37°C for 1 h and the absorbance at 340 nm measured by microtiter plate reader.

2.13 Double immunodiffusion analysis.

Double immunodiffusion analysis of the immunoreactivity of anti holoLTP and anti human apolipoprotein B sera with HDLp-W1, LDL, and LTP was carried out according to Ouchterlony (1968) in a 1 % agarose/PBS gel. Each of the anti sera (11 μ l) were challenged with LTP (10 μ g), HDLp-W1 (13 μ g), and human LDL (5 μ g) and after thorough washing with PBS and distilled water, the gel was stained with Coomassie Blue, dried, and photographed.

2.14 Immunoprecipitation experiments.

LTP (2 μ g) or PBS were preincubated with 2 μ l rabbit non-immune or anti holoLTP serum in a total volume of 90 μ l at 4°C for 60 min with shaking on a IKA-Vibrax-VXR (Janke & Kunkel) at 1000 rpm. Protein A Sepharose in 3 % bovine serum albumin/PBS was added at greater than twice the binding capacity required for IgG present. The samples were then incubated for a further 60 min at 4°C with shaking. Immune complexes were pelleted by centrifugation at 12 000 g for 3 min and an 100 μ l aliquot of the supernatant was assayed for LTP activity as described above.

LTP was radioiodinated to a specific activity of 3.3×10^4 cpm/ μ g with Iodogen beads as described above. Immunoprecipitation reactions with ^{125}I -LTP were carried out essentially as described by Harlow and Lane (1988). Immune complexes were formed by incubation of 0.6 μ g ^{125}I -LTP, 0.4 μ g LTP as a carrier, and 100 μ g non-immune, anti holoLTP, anti apoLTP-I, anti apoLTP-II, or anti apoLTP-III IgG for 1 h on ice. Protein-A Sepharose (80 μ l of 1 : 1 suspension with PBS) was added and the samples were incubated for 2 h at 4°C with shaking. The tubes were centrifuged at 12 000 g for 3 min, the supernatant removed, and the beads thoroughly washed with PBS. Supernatants and beads were treated with sample treatment buffer (8 M urea, 2 % SDS, 10 % glycerol, 5 % β -mercaptoethanol) and boiled 15 min prior to 4 - 12 % linear acrylamide gradient SDS-PAGE. The gel was dried and autoradiographed.

Detergent-containing immunoprecipitation experiments were carried out as described above except the immune complex reactions were carried out with 0.5 % NP-40, the Protein-A Sepharose was in a 1 : 1 suspension with 1 % NP-40/PBS, and the beads were washed with 1 % Tween-20/PBS.

2.15 NP-40 gel filtration of ^{125}I -LTP and LTP.

^{125}I -LTP (specific activity = 5.4×10^4 cpm/ μ g; 0.3 mg) was incubated with either 1 % NP-40 or PBS for 1 h on ice and then 3 h with shaking at 4°C and stored at 4°C until use. ^{125}I -LTP treated with 1 % NP-40 was applied to a Sepharose CL 6B column (183 ml) and eluted in 1.2 ml fractions with 1 % NP-40/PBS. ^{125}I -LTP preincubated with PBS was similarly subjected to gel filtration chromatography with an identical column except PBS was used as the elution buffer. Aliquots (50 μ l) of the column fractions were counted on a

gamma counter (Beckman Gamma 500) and 4 - 12 % linear acrylamide gradient SDS-PAGE gels were run on selected radioactivity-containing fractions (50 μ l per fraction). The gel was dried and phosphorimaged by a Fujix BAS 1000 phosphorimager with a BAS 1000 Bio-Imaging Analyzer.

2.16 Electron microscopy of NP-40 treated LTP and ^{125} I-LTP.

Electron microscopic studies were carried out in collaboration with Dr. Doug Scraba. LTP and ^{125} I-LTP were treated with 0.1 % NP-40/PBS or PBS for 1 h at 4°C, diluted to 20 μ g/ml with PBS and subjected to electron microscopy as described previously (Ryan *et al.*, 1990b). Samples were placed onto hydrophilic carbon film (prepared by glow discharge), which was supported by a parlodion film on a 300-mesh copper grid, washed with reticulocyte standard buffer (10 mM NaCl/1.5 mM MgCl₂/10 mM Tris-HCl pH 7.0), negatively stained with 2 % sodium phosphotungstate pH 7.0, and examined under a instrumental magnification of 74 400 with a Philips EM420 electron microscope operated at 100KV.

2.17 Effect of NP-40 on LTP and ^{125} I-LTP activity.

The lipid transfer activity of LTP and ^{125} I-LTP in the presence and absence of NP-40 was examined. LTP and ^{125} I-LTP in increasing amounts from 0.2 to 2.5 μ g were preincubated in microtiter plate wells with 0.5 % NP-40 or PBS for 1 h or were treated with 0.5 % NP-40 immediately before transfer assay. Turbidimetric lipid transfer assays were performed by incubating preincubated LTP or ^{125} I-LTP or that treated with 0.5 % NP-40 without incubation with 500

μ g HDLp-W1 protein and 100 μ g LDL protein for 45 min at 37°C. Transfer activity was measured by determining the absorbance at 340 nm as described above.

2.18 Circular dichroism spectroscopy.

Circular dichroism (CD) spectroscopy and analytical ultracentrifugation experiments were carried out in collaboration with Dr. Cyril Kay. The denaturation of LTP by increasing amounts of guanidine hydrochloride was monitored by CD spectroscopy. LTP (1.2 mg) was dialyzed into 50 mM NaH_2PO_4 /100 mM KCl pH 7.2, aliquots were treated with varying guanidine hydrochloride concentrations (0 to 7.3 M), and the CD spectra was obtained by Kim Oikawa on a Jasco J-500C spectropolarimeter with DP500N data processor and thermostated cell-holder with a waterbath at 25°C as described previously (Ryan *et al.*, 1990a). The cells used were 0.05 cm in the far-UV and 1 cm in the near-UV and sample size was 100 μ l. A guanidine hydrochloride denaturation concentration curve was constructed from these results.

2.19 Sedimentation velocity analytical ultracentrifugation experiments.

The dissociation of LTP into substituent apoproteins was examined by analytical ultracentrifugation experiments. LTP was dialyzed into 10 mM Tris-HCl/500 mM KCl pH 8.0, 10 mM Tris-HCl/500 mM KCl/3 M guanidine hydrochloride pH 8.0, 10 mM Tris-HCl/500 mM KCl/4.5 M guanidine hydrochloride pH 8.0, or 10 mM Tris-HCl/500 mM KCl/6 M guanidine

hydrochloride pH 8.0, 1.4 mg LTP each, for 48 h. Sedimentation velocity analytical ultracentrifugation experiments at 60 000 rpm at 10°C on a Beckman model E analytical ultracentrifuge with an electronic speed control system, RIRC temperature control system, and titanium rotor were performed by Mr. Leslie Hicks. Schlieren (Schlieren optical system) photographs were taken and measurements of photographic plates were performed on a Nikon model 6 microcomparator as described previously (Ryan *et al.*, 1990a). Mr. Hicks calculated apparent $s_{20,w}$ values for the leading peak in each schlieren pattern correcting for the viscosity of the guanidine hydrochloride solutions according to Chervenka (Chervenka, 1970; Freifelder, 1982). Equations used in this calculation were:

Equation 1:

$$s = \frac{1}{\omega^2 r} \frac{dr}{dt}$$

Equation 2:

$$s_{20,w} = s_{\text{obs}} \left(\frac{\eta_t}{\eta_{20}} \right) \left(\frac{\eta_{\text{sol}}}{\eta_w} \right) \left(\frac{1 - \bar{v}\rho_{20,w}}{1 - \bar{v}\rho_T} \right)$$

where: s = sedimentation coefficient

$1/\omega^2 r$ = run constant, ω^2 = angular velocity, r = rotor radius

dr/dt = rate of boundary movement

$s_{20,w}$ = standard sedimentation coefficient

η_t/η_{20} = relative viscosity of water at temperature T compared with that at 20°C

η_{sol}/η_w = relative viscosity of the solvent to that of water

$\bar{v}$ = partial specific volume

$\rho_{20,w}$ = density of water

ρ_T = density of solvent

2.20 Guanidine hydrochloride/DTT gel filtration chromatography of denatured LTP.

LTP (3.0 mg) was extensively dialyzed into PBS, solid guanidine hydrochloride was added to 8 M, DTT was added to 2 mM and denaturing gel filtration chromatography (Sephadex G-150 30 ml column) with 6 M guanidine hydrochloride/50 mM Tris-base/1 mM DTT pH 8.0 elution buffer was performed. The absorbance of each 1 ml fraction was measured at 280 nm and the fraction with the peak absorbance was dialyzed against 6 M urea and analyzed by 4 - 12 % linear acrylamide gradient SDS-PAGE.

2.21 SDS gel filtration chromatography of LTP.

Experimentation to achieve the isolation of individual LTP apoproteins was extended by SDS gel filtration chromatography. LTP (2.8 mg, dialyzed into PBS) was denatured with 2 % SDS and subjected to sequential SDS denaturing gel filtration chromatography. Denatured LTP was applied to an 183 ml Sepharose CL 6B column and 1 ml fractions were eluted with 2 % SDS/PBS and analyzed by measuring the absorbance at 280 nm and 4 - 12 % linear acrylamide gradient SDS-PAGE gel. Fractions enriched in either apoLTP-I or apoLTP-II were pooled, concentrated, and apoLTP-II-enriched fractions were subjected to another gel filtration chromatography step and analyzed by SDS-PAGE.

2.22 Amino acid analysis of LTP apoproteins from PVDF membrane.

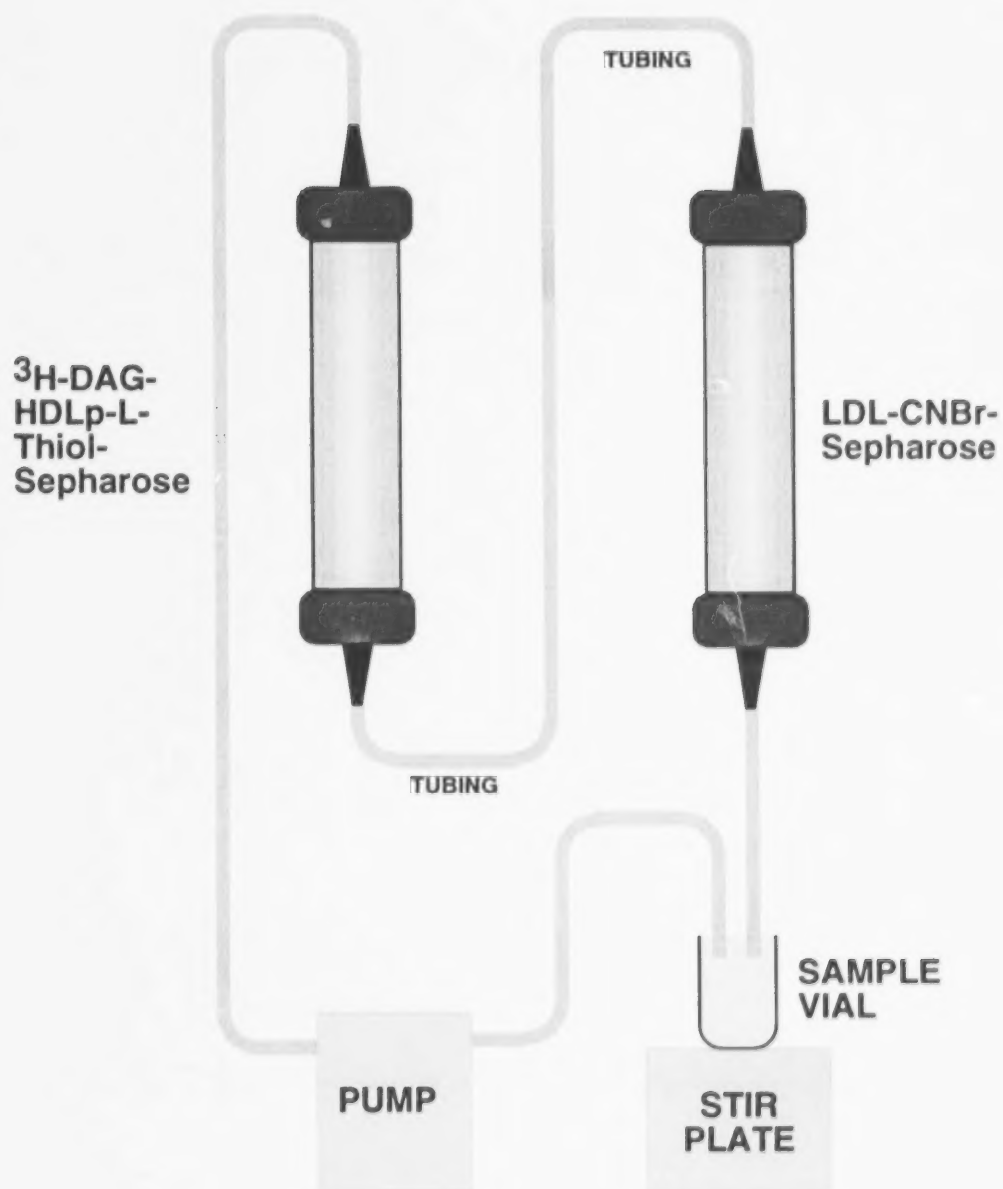
Attempts were made to determine the amino acid composition of LTP apoproteins. Purified LTP (658 μ g) was run on 4 - 15 % linear acrylamide gradient SDS-PAGE gel and the apoproteins transferred to PVDF membrane in 10 mM CAPS pH 11 at 150 mA for 16 h for apoLTP-II and apoLTP-III and 20 h for apoLTP-I. Membrane slices containing LTP apoproteins were subjected to hydrolysis at 6N HCl at high temperature and the amino acids analyzed by Alberta Peptide Institute. Tryptophan and cysteine residues were not analyzed by this method. Tryptophan-specific and cysteine-specific hydrolysis conditions did not hydrolyze the residues from the PVDF immobilized protein.

2.23 Trypsin digestion of LTP and partial amino acid sequence determination.

LTP (35 μ g) was dialyzed into PBS and trypsin digested with 0.375 μ g trypsin by incubation at room temperature for 1 h. The trypsin reaction was stopped by the addition of soybean trypsin inhibitor in a two-fold molar excess over trypsin and sample treatment buffer. Trypsin-digested LTP was run on 4 - 15 % linear acrylamide gradient SDS-PAGE gel and transferred to PVDF in 10 mM CAPS pH 11. The membrane was stained with Coomassie Blue and bands were excised and microsequenced by Mr. Mike Carpenter. Immunoblot analysis confirmed that the apoLTP-II fragment arose from apoLTP-II.

Figure 2.1 Circulation system utilized in mechanistic studies.

Two columns packed with [^3H]DAG-HDLp-thiol-Sepharose, LDL-CNBr-Sepharose, or Sepharose 4B were connected in series with tubing, a pump to circulate buffer and an open reservoir to allow the addition of proteins.



CHAPTER 3

STUDIES OF THE MECHANISM OF ACTION OF LIPID TRANSFER PARTICLE WITH SOLID-PHASE LIPOPROTEIN SUBSTRATES

a version of this chapter has been previously published:

Blacklock, B. J., Smillie, M., Ryan, R. O. (1992) Insect lipid transfer particle can facilitate net vectorial lipid transfer via a carrier-mediated mechanism. *J. Biol. Chem.* **267**, 14033-14037.

3.1 Introduction.

Numerous *in vitro* studies of lipid and lipid particle substrate specificity of LTP were described in the introduction of this dissertation. Briefly, lipoproteins including insect lipophorins (Ryan *et al.*, 1988b,c; Ryan *et al.*, 1990c,d; Singh and Ryan, 1991), human HDL (Silver *et al.*, 1990) and LDL (Ryan *et al.*, 1990d), and apolipoprotein-stabilized TAG/phosphatidylcholine microemulsions (Ando *et al.*, 1990) serve as substrates for LTP-mediated lipid transfer. Lipids which have been shown to be transferred by LTP include DAG (Ryan *et al.*, 1988b,c; Ryan *et al.*, 1990d; Singh and Ryan, 1991; Singh *et al.*, 1992b), long chain aliphatic hydrocarbons (Singh and Ryan, 1991; Takeuchi and Chino, 1993), TAG, and PL (Ando *et al.*, 1990; Singh *et al.*, 1992b). The size of the LTP particle, its lipid content, and general lack of specificity for lipid substrates suggests LTP has an exchangeable and transferrable lipid pool rather than a specific lipid binding site. Previous studies have shown that the lipid component of *M. sexta* LTP is in dynamic equilibrium with lipids associated with donor and acceptor lipoproteins since incubation of [^{3} H]DAG-HDLp-L with substrate quantities of LTP resulted in the association of [^{3} H]DAG with LTP. This [^{3} H]DAG was subsequently transferred back to unlabelled lipophorin (Ryan *et al.*, 1988c). Attempts to deplete LTP of its lipid component, through organic extraction and detergent solubilization, resulted in a loss of lipid transfer activity (Ryan *et al.*, 1988c; Ryan *et al.*, 1990b). Reconstitution of LTP with nitrocellulose-bound apoproteins did not restore transfer activity (Ryan *et al.*, 1988c). These results suggest that the lipid component of LTP is involved in lipid transfer and does not act only as a structural component of the particle.

LTP facilitates net vectorial transfer of HDLp-L-associated DAG to human LDL in solution. This transfer is linearly dependent on time and LTP concentration. Exchange or transfer of apolipoproteins among substrate particles does not occur. When a 1 : 1 protein ratio of lipophorin : LDL is present, LTP mediates redistribution of up to 90% of the DAG from lipophorin to LDL in the absence of significant reciprocal net lipid transfer from LDL to lipophorin (Ryan *et al.*, 1990d). DAG transfer to LDL, therefore, constitutes facilitated net vectorial mass transfer and, at equilibrium, produces a lipid-enriched LDL and a relatively lipid-depleted lipophorin. The rate and magnitude of this facilitated net transfer makes this donor/acceptor lipoprotein pair excellent candidates for mechanistic studies.

The mechanism of facilitated lipid transfer by insect or mammalian plasma transfer catalysts is not understood. Two possible mechanisms proposed for mammalian cholesteryl ester transfer protein (reviewed by Morton, 1990 and Tall, 1993) apply equally well to insect LTP-mediated transfer. Lipid transfer may proceed via a ternary complex whereby the transfer catalyst interacts with donor and acceptor lipid particles simultaneously to effect transfer (Figure 3.1). Alternatively, the transfer catalyst may act as a carrier of lipid by acquiring lipid from a donor lipoprotein, carrying it through the aqueous medium, and subsequently delivering it to an acceptor lipoprotein (Figure 3.1). Attempts have been made to distinguish between these mechanisms through kinetic studies of CETP (Barter and Jones, 1980; Ihm *et al.*, 1982) and the isolation of possible reaction intermediates in which CETP has bound transferrable lipid (Swenson *et al.*, 1988). These studies were contradictory in their conclusions as to the mechanism of action of CETP. The mechanism of lipid transfer for the intracellular microsomal triacylglycerol

transfer protein complex (MTP) has also been examined through kinetic analysis and equilibrium gel filtration chromatography (Atzel and Wetterau, 1993). Kinetic studies revealed that MTP utilizes a ping-pong bi-bi mechanism which suggests that MTP shuttles between donor and acceptor vesicles as in a carrier-mediated model.

The mechanism of action of insect LTP has not been established although the demonstration that the DAG component of LTP is exchangeable with substrate lipoproteins supports a carrier-mediated mechanism. The problem of determining the mechanism of action of LTP was approached with a novel system in which substrate lipoproteins were physically separated by covalent linkage to Sepharose matrices. LTP was circulated between solid-phase lipoprotein substrates and transfer of DAG analyzed. The contribution of ternary complex formation to LTP transfer activity was examined by circulating each of the substrate lipoproteins individually with LTP while the other remained immobilized. The results demonstrate that LTP can catalyze net vectorial lipid transfer via a carrier-mediated mechanism.

3.2 Results.

In this study, [^3H]DAG-HDLp-L and LDL were physically separated by covalent linkage to Sepharose beads and packing into separate columns connected in series. LTP or buffer were circulated between the columns and the extent of [^3H]DAG transfer from lipophorin to LDL was determined after 16 hours. LTP mediated [^3H]DAG transfer from HDLp-L to LDL in a linear, LTP concentration dependent manner (Figure 3.2). [^3H]DAG did not transfer to LDL to a significant extent in the absence of LTP since greater than 83 % of the counts

originally associated with [^3H]DAG-HDLp-L were recovered with the washed [^3H]DAG-HDLp-L-thiol-Sepharose. HDLp-L retained its apoprotein composition upon binding and circulation of PBS or LTP, as judged by SDS-PAGE of eluted lipophorin. Furthermore, when ^{125}I -apolipoprotein B-LDL was bound to CNBr-Sepharose, greater than 96 % of the total ^{125}I counts recovered were associated with LDL-CNBr-Sepharose when PBS or LTP were circulated. These results verify that the lipoprotein particles remained fixed to the solid matrix.

To determine the specificity of the DAG transfer reaction, an unrelated *M. sexta* hemolymph storage protein, arylphorin, was substituted for LTP in solid-phase transfer assays. Only 2 - 3 % of the recovered label was found associated with LDL-CNBr-Sepharose when arylphorin or PBS were circulated (Table 3.1). In contrast, when LTP was present, 26 % of the total recovered counts were associated with the LDL-CNBr-Sepharose.

The observed rates of facilitated and spontaneous transfer when donor and acceptor lipoproteins were physically separated are shown in Table 3.1. Transfer of DAG in the absence of LTP, which corresponds to spontaneous transfer via aqueous diffusion, was negligible. Diacylglycerol transfer occurred at a 14-fold greater rate in the presence of LTP than in the absence of LTP. The specific rate of DAG transfer (10.4 ng DAG/h/mg LTP), determined by subtraction of spontaneous transfer from total transfer, can be attributed to LTP acting as a carrier.

The effect of circulating acceptor LDL on lipid transfer from solid-phase HDLp-L was examined in the presence and absence of LTP. The same assay system was used with Sepharose CL 4B substituting for LDL-CNBr-Sepharose.

Figure 3.3 shows that [^3H]DAG transferred to unbound LDL in the presence, but not the absence, of LTP when circulation was followed by density gradient ultracentrifugation of the circulation media. Radioactivity was recovered at a density corresponding only to that of LDL in the presence or absence of LTP confirming that labelled HDLp-L remained bound to the thiol-Sepharose matrix. Table 3.2 shows the rate of transfer of DAG from bound [^3H]DAG-HDLp-L to circulating LDL in the above experiment. The rate of DAG transfer in the presence of LTP was 13-fold greater than in the control experiment lacking LTP. DAG transfer in the absence of LTP represents the combined contribution of transfer via lipoprotein collision and by aqueous diffusion. Under these conditions the rate of specific transfer of DAG by LTP increased to 75.0 ng DAG/h/ μg LTP. Similar results were obtained when LDL was bound to the solid-phase and [^3H]DAG-HDLp-L was circulated (Table 3.3). The rate of DAG transfer in the presence of LTP was 12-fold greater than in the control experiment lacking LTP where transfer occurred by the combined effects of lipoprotein collision and aqueous diffusion. The specific rate of transfer when the donor lipoprotein was circulated and the acceptor was immobilized was 65.0 ng DAG/h/ μg LTP, a value similar to that found when the acceptor lipoprotein was circulated.

The possibility that the increase in transfer rate when one of the lipoprotein substrates were circulated may be due to increased access of LTP to acceptor lipoprotein was examined by circulating both LDL and HDLp-L in the presence and absence of LTP. In this case, LTP induced an 8-fold enhancement in the rate of DAG transfer from 13.9 to 113 μg DAG/h, corresponding to a specific transfer rate of 2.26 μg DAG/h/ μg LTP.

3.3 Discussion.

Possible mechanisms by which lipid may be transported between lipoproteins include 1) transfer by specialized proteins, 2) collision of donor and acceptor lipoproteins with transport at the point of contact, and 3) passive diffusion through the aqueous medium. In this study, the mechanism of facilitated lipid transfer by *M. sexta* LTP has been examined. Transfer proteins may facilitate lipid transfer through a ternary complex or by acting as a carrier of lipid between lipoproteins or between lipoproteins and membranes (Figure 3.1). The work presented here was designed to assess the ability of LTP to transfer DAG from [^3H]DAG-HDLp-L to LDL via a carrier-mediated mechanism and whether ternary complex formation contributes to LTP-mediated transfer.

In control experiments with both substrate lipoproteins immobilized, DAG transfer may occur only via diffusion through the aqueous medium. After 16 h of buffer circulation, less than 1 % of the HDLp-L-associated [^3H]DAG was recovered with solid-phase LDL. This result demonstrates the stability of the solid-phase lipoproteins employed as well as the relative hydrophobicity of DAG which prevents significant spontaneous transfer of [^3H]DAG. By contrast, circulation of LTP between the lipoproteins induced DAG transfer from HDLp-L to LDL in a concentration dependent manner (Figure 3.2). This result establishes that LTP is capable of utilizing a carrier-mediated mechanism of lipid transfer. When acceptor LDL particles were circulated, spontaneous transfer in the absence of LTP was found to contribute less than 8 % of facilitated [^3H]DAG transfer. Under these conditions, spontaneous DAG transfer may occur via aqueous diffusion (Pownall *et al.*, 1983; Charlton *et al.*, 1978) or through binary collision between lipoproteins. Increased DAG transfer was observed when the acceptor lipoprotein was circulated with LTP

while the donor lipoprotein was immobilized (Table 3.2). Similarly, increased DAG transfer was observed when [^3H]DAG-HDLp-L was circulated and LDL remained bound to a Sepharose matrix (Table 3.3). Under these conditions, LTP has the opportunity to act as a carrier of lipid or mediate a productive interaction between donor and acceptor lipoproteins. Thus, the observed increased DAG transfer may be a reflection of a ternary complex mechanism, enhanced carrier action due to the shorter distances LTP-lipid complexes would be required to travel to effect transfer, increased substrate particle accessibility, or enhanced aqueous diffusion of lipid. The comparable LTP induced rate enhancement (12 to 14 - fold greater than the corresponding control) observed under conditions where both substrate lipoproteins were immobilized and when only one was bound suggests the contribution of ternary complex formation to lipid transfer is insignificant. In the latter case, an additive effect would be expected if a ternary complex mechanism contributed to transfer.

The rates of diacylglycerol transfer discussed above were determined with either one or both lipoproteins covalently bound to a Sepharose matrix. The transfer experiment was also carried out with the circulation system packed with Sepharose CL 4B and substrate lipoproteins circulated in the presence and absence of LTP. The specific rate of transfer under these conditions was 2.26 $\mu\text{g DAG/h}/\mu\text{g LTP}$, corresponding to an 8-fold rate enhancement versus the control experiment. The fact that this rate is far greater than that found when one or both lipoproteins were bound indicates that binding of the lipoproteins to Sepharose may hinder access of LTP to substrates. The increase in transfer rate when both lipoproteins were unbound may also be a result of increased opportunity for transfer to occur. When one or both lipoproteins were bound, the transfer reaction was restricted to the immediate vicinity of the lipoprotein-

Sephadex matrices. However, when all three participants were circulated, the reaction could occur throughout the system. A consequence of these factors would be an under-estimation of the rate of carrier-mediated transfer determined with both lipoproteins bound.

The specific rate of transfer of DAG from HDLp-L to LDL in solution is 144 $\mu\text{g DAG/h}/\mu\text{g LTP}$ determined at 33°C in 0.5 ml volume (Ryan *et al.*, 1990d). This rate is 60 times greater than the rate found with both lipoproteins free in the circulation system used in this study. The difference likely results from the effect of dilution in the circulation system, the lower temperature at which the present studies were conducted, hindered accessibility of LTP to lipoprotein substrates, or a combination of these.

An intermediate of carrier-mediated lipid transfer by LTP has been previously isolated. When substrate quantities of LTP were incubated with [^3H]DAG-HDLp-L, [^3H]DAG transferred from the lipoprotein to LTP and [^3H]DAG-labelled LTP was isolated. The [^3H]DAG subsequently transferred from LTP to unlabelled HDLp-L (Ryan *et al.*, 1988c) supporting the conclusion that LTP is capable of acting as a carrier of lipid in the transfer reaction.

The mechanism of action of the human plasma CETP has been the subject of controversy for over a decade. The problem has been examined by kinetic studies and through the isolation of possible reaction intermediates in which CETP binds lipid or lipid particle substrates. Transfer via a ternary complex is suggested by kinetic studies where Ihm *et al.* (1982) found that a ternary complex mechanism is favoured if CETP is initially bound to a lipoprotein substrate particle. Evidence for this binding was found in at least four studies. Morton (1985) showed that CETP binds to plasma lipoprotein substrates, LDL,

VLDL, and more stably, HDL. Enhanced CETP binding and activity was observed with lipoprotein lipase-treated VLDL and HDL substrates (Sammett and Tall, 1985). CETP has also been shown to bind phosphatidylcholine vesicles containing either TAG or CE in experiments where CETP was incubated with vesicles and binding of lipid and/or vesicles was analyzed by gel filtration chromatography (Swenson *et al.*, 1988). CETP binding to phospholipid vesicles has also been demonstrated (Hesler *et al.*, 1987). Other kinetic evidence also supports a carrier-mediated mechanism of transfer for CETP (Barter and Jones, 1980). Possible reaction intermediates of a carrier mediated mechanism were found by Swenson and coworkers. Scatchard analysis suggested that CETP has a single class of binding sites for both CE and TAG with a stoichiometry of binding of 0.9 mol CE/mol CETP and 0.2 mol TAG/mol CETP (Swenson *et al.*, 1988). CETP, with bound CE and TAG donated by phosphatidylcholine vesicles, was isolated and this neutral lipid could be transferred to LDL (Swenson *et al.*, 1988). The isolation of a transfer protein with bound lipid does not necessarily rule out ternary complex transfer since the transfer protein may have obtained lipid while catalyzing transfer via a ternary complex and the isolation procedure may have disrupted this interaction.

Subsequent to the publication of the work described in this chapter, Atzel and Wetterau (1993) reported that MTP displays ping pong bi-bi kinetics which may correspond to a carrier-mediated mechanism. This mechanism was supported by lipid binding experiments in which MTP was shown to bind pyrene-labelled CE (Atzel and Wetterau, 1993). Purified bovine MTP was also found to contain bound lipid (Wetterau and Zilversmit, 1985). The fact that MTP is an endoplasmic reticulum luminal protein which does not associate

with the membrane (Wetterau and Zilversmit, 1986) supports a carrier-mediated mechanism of action. If MTP were a membrane-associated protein, transfer via a ternary complex mechanism would be more likely.

LTP can modify the DAG content of pre-existing lipophorin (Ryan *et al.*, 1986a) and has been shown to promote lipid loading from fat body to lipophorin *in vitro* during adipokinetic hormone-induced diacylglycerol mobilization (Van Heusden and Law, 1989). LTP may also play a role in the loading of lipid in *M. sexta* oocyte where it is proposed to induce transformation of HDLp-A to VHDLp-E (Liu and Ryan, 1991). The carrier-mediated mechanism of action of LTP demonstrated in this study may have a physiological role in the interconversion of lipophorin subspecies, loading of DAG from fat body to lipophorin, and from lipophorin into the egg (Kawooya and Law, 1988; Liu and Ryan, 1991).

Figure 3.1 Alternative mechanisms of action of lipid transfer catalysts.

Lipid transfer catalysts can facilitate lipid transfer by either the carrier-mediated or ternary complex mechanisms of action. In the carrier mediated mechanism, LTP acquires lipid from a donor particle, HDLp-L, shuttles it through the aqueous medium, and delivers it to the acceptor particle, LDL. Alternatively, LTP may mediate transfer through a ternary complex of LTP, HDLp-L, and LDL.

Carrier Mediated



Ternary Complex

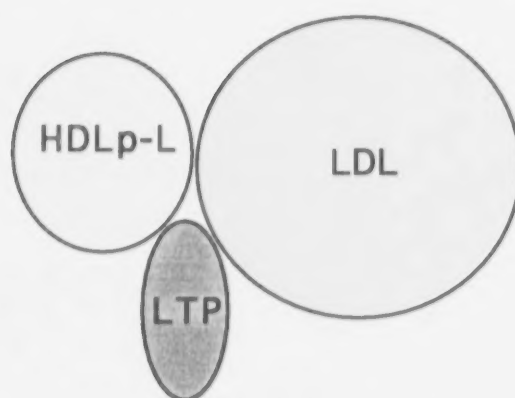


Figure 3.2 LTP concentration dependence of [³H]DAG transfer between solid-phase lipoproteins.

Separate 2 ml columns were packed with 2.5 mg [³H]DAG-HDLp-L-thiol-Sepharose protein (specific activity = 1.1×10^5 cpm/mg protein) and 5 mg LDL-CNBr-Sepharose protein, respectively. The columns were connected in series and LTP was circulated in concentrations ranging from 0 to 100 mg/ml for 16 hours at 23°C. The [³H]DAG recovered in each column was measured as described in Chapter 2 and plotted as % recovered cpm versus LTP concentration (mg/ml), open circles, HDLp-L; closed circles, LDL.

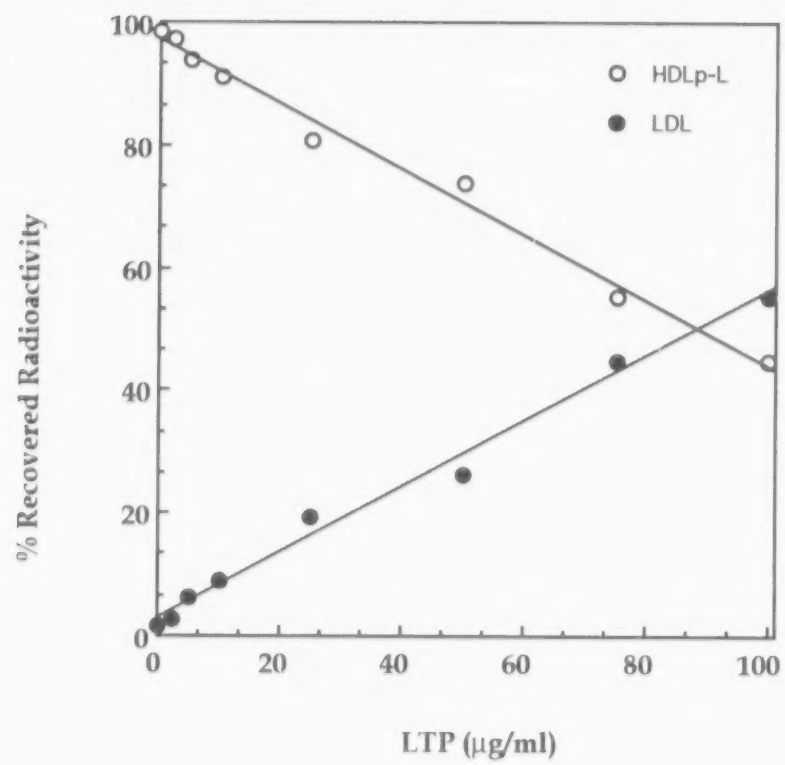


Table 3.1 Specificity of LTP-dependent carrier-mediated [^3H]DAG transfer between solid-phase lipoproteins.

Separate 2 ml columns were packed with [^3H]DAG-HDLp-L-thiol-Sepharose (0.6 mg [^3H]DAG-HDLp-L protein; specific activity = 4.4×10^5 cpm/mg protein) and LDL-CNBr-Sepharose (1.0 mg LDL protein), respectively. LTP (25 mg/ml), arylphorin (25 mg/ml), or PBS were circulated for 16 hours at 23°C. The [^3H]DAG recovered in each column was measured and rate calculations were carried out as described in Chapter 2. The results presented are representative of experiments performed on 4 separate occasions with slight modifications. LTP induced transfer of 22.3 ± 7.5 % (mean $\pm$ S.D.; n=4) HDLp-L-associated radioactivity to LDL versus 2.5 ± 0.6 % (n=4) in control experiments.

	Recovered cpm		rate of transfer (μg DAG/h)	specific transfer (ng DAG /h/ μg LTP)
	HDLp-L	LDL		
no addition	221 054	4 118	0.16	-----
+ arylphorin	233 264	6 493	0.25	-----
+LTP	167 466	57 903	2.24	10.4

Figure 3.3 LTP-dependent transfer of [^{3} H]DAG from solid-phase lipophorin to circulating LDL.

Separate 2 ml columns were packed with [^{3} H]DAG-HDLp-L-thiol-Sepharose (0.6 mg [^{3} H]DAG-HDLp-L protein; specific activity 4.4×10^5 cpm/mg protein) and Sepharose CL 4B, respectively. LTP (25 mg/ml) (closed circles) or PBS (open circles) and LDL (1.0 mg LDL protein) were circulated for 4 hours at 23°C. The LDL was collected, subjected to density gradient ultracentrifugation, and analyzed as described in Chapter 2. cpm/50 μ l aliquot is plotted versus density (g/ml).

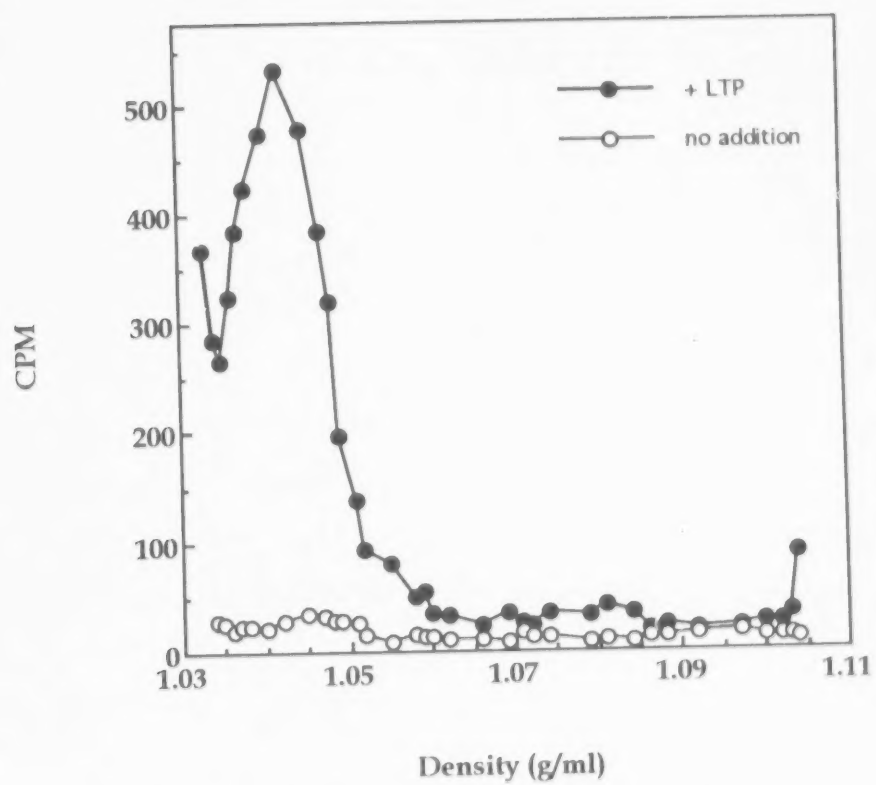


Table 3.2 Effect of circulation of acceptor lipoprotein on LTP-dependent [^{3} H]DAG transfer between lipoproteins.

Separate 2 ml columns were packed with [^{3} H]DAG-HDLp-L-thiol-Sepharose (0.6 mg [^{3} H]DAG-HDLp-L protein; specific activity 4.4×10^5 cpm/mg protein) and Sepharose CL 4B, respectively. LDL (1.0 mg LDL protein) was circulated for 4 hours at 23°C in the presence and absence of LTP (25 mg/ml). The [^{3} H]DAG recovered in each column was measured and rate calculations were carried out as described in Chapter 2. The results presented are representative of experiments performed on 3 separate occasions with slight modifications. LTP induced transfer of 39.3 ± 7.5 % (mean $\pm$ S.D.; n=3) of HDLp-L-associated radioactivity to LDL versus 4.6 ± 1.3 % (n=3) in control experiments.

	Recovered cpm		rate of transfer (μ g DAG/h)	specific transfer (ng DAG /h/ μ g LTP)
	HDLp-L	LDL		
no addition	213 898	8 234	1.3	-----
+ LTP	114 464	105 340	16.3	75.0

CHAPTER 4

STRUCTURE-FUNCTION STUDIES OF LIPID TRANSFER PARTICLE WITH APOPROTEIN-SPECIFIC ANTIBODIES

Table 3.3 Effect of circulation of donor lipoprotein on LTP-dependent [^3H]DAG transfer between lipoproteins.

Separate 2 mL columns were packed with Sepharose CL 4B and LDL-CNBr-Sepharose (1.0 mg LDL protein), respectively. [^3H]DAG-HDLp-L (0.6 mg [^3H]DAG-HDLp-L protein: specific activity = 4.4×10^5 cpm/mg protein) was circulated in the presence and absence of LTP (25 $\mu\text{g}/\text{ml}$) for 4 h at 23°C. The [^3H]DAG recovered in the LDL-CNBr-Sepharose column was measured and rate calculations were carried out as described in Chapter 2. The results presented are representative of experiments performed on 2 separate occasions with slight modifications. LTP induced transfer of 35.3 and 36.4 % of HDLp-L-associated radioactivity to LDL-CNBr-Sepharose in the two experiments versus 4.1 and 5.8 % in control experiments.

	Recovered cpm		
	LDL	rate of transfer (μg DAG/h)	specific transfer (ng DAG /h/ μg LTP)
no addition	10 774	1.3	---
+ LTP	92 114	14.3	65.0

4.1 Introduction.

The large size, unusual morphology, presence of lipid and three apoproteins, and lipid transfer ability of LTP pose intriguing questions about the structure, function, and activity relationships of the particle and makes LTP unique among plasma lipid transfer catalysts. The mammalian plasma lipid transfer catalysts, cholesteryl ester transfer protein and phospholipid transfer protein, are responsible for the exchange of triacylglycerol, cholesteryl ester, and phospholipids between lipoproteins within the plasma (Tall, 1993). Both proteins are monomeric and have approximate M_r of 74 000 (Tall, 1993) and 70 000 (Tollefson *et al.*, 1988), respectively. Generally, intracellular lipid transfer catalysts are small, monomeric polypeptides (Helmkamp, 1986) with the exception of the liver and intestinal MTP (Wetterau *et al.*, 1990). In contrast, LTP has been shown to possess three apoproteins; apoLTP-I, apoLTP-II, and apoLTP-III comprising 86 % of the particle mass and is 14 % lipid by weight for a particle molecular mass of greater than 1×10^6 Da (Ryan *et al.*, 1988c). This has led to speculation that the smaller LTP apoproteins are proteolytic products of apoLTP-I or that copurification of multiple proteins is occurring.

LTP catalyzes the net transfer or exchange of a number of lipids among a variety of lipid particle substrates (for review see: Blacklock and Ryan, 1993). The lipid component of LTP is essential for lipid transfer (Ryan *et al.*, 1988c). Chapter 3 demonstrated that LTP can utilize a carrier-mediated mechanism in lipid transfer. The apoprotein(s) responsible for the lipid transfer ability of LTP, however, are not known. Neither the physiological and biochemical importance of the unique morphology nor the spatial relationships of the apoproteins within the particle have been defined. Previous work has

suggested that apoLTP-III is buried within the particle and apoLTP-I and apoLTP-II are exposed (Ryan *et al.*, 1990b) but examination by a different experimental means is required.

Antibodies have been employed as a tool to examine structure and function of many proteins. This chapter describes studies of LTP structure and function utilizing polyclonal antibodies raised against each of the LTP apoproteins. When this project was initiated, SDS-PAGE was the only known method to separate LTP apoproteins. Antibodies were, therefore, raised against apoproteins from gel slices. These antibodies were characterized by immunoblot analysis and enzyme linked immunosorbent assays (ELISA). They were then used to examine the role of the apoproteins in lipid transfer and in immunoprecipitation experiments. The results obtained provide new information on the immunorelationship between apoproteins, the identity of a catalytically important apoprotein, and the structural organization of the particle.

4.2 Results.

4.2.1 Immunological relatedness of LTP apoproteins.

LTP has previously been shown to be immunologically distinct from other hemolymph components (Ryan *et al.*, 1988b). In this study, antibodies raised against each of the LTP apoproteins were used to examine the immunological relatedness of the apoproteins and the specificity of the antibodies was determined. Figure 4.1 is an immunoblot of purified LTP probed with anti sera against holoLTP (lane 1), apoLTP-I (lane 2), apoLTP-II

(lane 3), apoLTP-III (lane 4) and non-immune serum (lane 5). Anti holoLTP recognized all three apoproteins while each of the antisera raised against a particular apoprotein immunoreacted with only the apoprotein against which it was raised. There was no cross-reactivity between non-immune serum and LTP.

4.2.2 Characterization of LTP apoprotein-specific IgG by ELISA.

The ability of the apoprotein-specific antibodies to recognize holoLTP was examined by antibody-capture ELISA. This was necessary because the antibodies were raised against denatured apoproteins from SDS-PAGE gel slices. Figure 4.2 illustrates the IgG concentration dependence of immunoreactivity of each of the apoprotein-specific antibody populations to LTP bound to a microtiter plate. These results show the apparent affinity with which each of the antibody populations bind LTP under these conditions. Anti apoLTP-I binds LTP with the greatest apparent affinity having a half maximum binding value of 2 ng IgG/ μ l. Anti apoLTP-II and anti apoLTP-III bind LTP with a half maximum binding value of 9 and 20 ng IgG/ μ l, respectively. There was a slight increase in absorbance at 405 nm at higher concentrations of control IgG (irrelevant anti larval serum protein IgG) due to non-specific reactivity in the assay. In order to verify that the apoprotein-specific antibodies recognize LTP in solution, antigen competition antibody capture ELISA experiments were carried out. Figure 4.3 shows the results from the antigen competition experiment in which an 100-fold excess of soluble LTP versus LTP bound to the well was added with the primary antibody. These results demonstrate that LTP in solution will compete entirely for the binding of all

three apoprotein-specific antibodies. Together these results demonstrate that each of the apoprotein-specific antibody preparations bind native LTP with differing apparent affinities.

ELISA experiments were also carried out in the presence of 0.05 % Tween-20 as per standard protocols. The IgG concentration dependence of immunoreactivity under these conditions is shown in Figure 4.4. The half maximal binding values interpolated from this graph are for anti apoLTP-I; 2 ng IgG/ μ l, anti apoLTP-II; 10 ng IgG/ μ l, and anti apoLTP-III; 10 ng IgG/ μ l. As in the non-detergent containing assays, there was a slight increase in absorbance at 405 nm at high concentrations of control IgG due to non-specific reactivity.

4.2.3 Inhibition of LTP-mediated DAG transfer by apoprotein-specific antibodies.

LTP apoprotein-specific antibodies were used to examine the role of individual apoproteins in the catalysis of lipid transfer by LTP. Immune complexes of LTP and anti apoLTP-I, anti apoLTP-II, and anti apoLTP-III were assayed for transfer activity by a turbidimetric assay (Singh *et al.*, 1992a). Figure 4.5 shows that each of the apoprotein-specific antibody preparations inhibited LTP activity. Anti apoLTP-II effectively inhibited transfer activity as demonstrated by the lack of sample turbidity at low IgG concentrations (20 μ g IgG) while anti apo-LTP-I and anti apoLTP-III IgG inhibited activity only when present in large amounts (greater than 100 μ g IgG). LTP activity was entirely inhibited by apoLTP-I at 400 μ g IgG while anti apoLTP-III inhibited less than 40 % of LTP transfer activity at this concentration. In control experiments, non-

immune IgG were unable to inhibit LTP-mediated lipid transfer activity. Incubation of non-immune IgG with substrate lipoproteins, in the absence of LTP, did not result in sample turbidity indicating facilitated DAG transfer did not occur (data not shown). Double immunodiffusion analysis (Figure 4.6) demonstrates that anti holoLTP immunoreacts with only LTP and not LDL or HDLp-W1. Similarly, anti human apolipoprotein-B immunoreacts with only LDL. The inhibition of transfer activity observed here was, therefore, not due to cross reactivity between substrate lipoproteins and LTP apoprotein-specific antibodies.

4.2.4 Immunoprecipitation experiments.

The immunoprecipitation of LTP from solution by anti holoLTP was examined by conducting turbidimetric transfer assays with post-immunoprecipitation supernatant. Figure 4.7 is a bar graph which demonstrates that anti holoLTP serum immunoprecipitates all of the lipid transfer activity from the supernatant of the reaction as compared to non-immune serum.

The spatial organization and stability of the LTP complex was also investigated with apoprotein-specific antibodies. LTP was radioiodinated and Figure 4.8, lane 1 demonstrates that each of the three apoproteins were radiolabelled. ^{125}I -LTP was employed in immunoprecipitation experiments with non-immune, anti holoLTP, anti apoLTP-I, anti apoLTP-II, and antiLTP-III IgG and Protein-A Sepharose under native conditions. There was a small amount of non-specific immunoprecipitation observed when non-immune IgG was used (Figure 4.8, lane 2). Anti holoLTP, anti apoLTP-I, and anti apoLTP-II

IgG were all able to immunoprecipitate ^{125}I -LTP (Figure 4.8, lanes 3, 4, 5) while anti apoLTP-III IgG were not (Figure 4.8, lane 6).

Immunoprecipitation experiments were also carried out under conditions where non-ionic, non-denaturing detergent was included in the immune complex reaction, precipitation by Protein-A Sepharose, and washing of the beads according standard protocol. Figure 4.9, panel A shows the immunoprecipitated material (pellets) when non-immune (lane 1), anti holoLTP (lane 2), anti apoLTP-I (lane 3), anti apoLTP-II (lane 4), and anti apoLTP-III (lane 5) IgG were used. Again, there was a small amount of non-specific immunoprecipitation with non-immune IgG. Anti holoLTP IgG immunoprecipitated all three ^{125}I -LTP apoproteins. Anti apoLTP-I and anti apoLTP-II, however, both immunoprecipitated only apoLTP-I and apoLTP-II. Panel B, lanes 3 and 4 show that apoLTP-III was not immunoprecipitated and remained in the supernatant of anti apoLTP-I and anti apoLTP-II reactions. Anti apoLTP-III IgG immunoprecipitated only apoLTP-III (Figure 4.9, panel A, lane 5) while apoLTP-I and apoLTP-II remained in supernatant (Figure 4.9, panel B, lane 5). Similar results were observed when the immunoprecipitation was carried out with 0.05 and 0.1 % NP-40 in the immune complex reaction.

4.3 Discussion.

Polyclonal antibodies specific to each of the LTP apoproteins were produced in rabbits upon injection of apoproteins separated by preparative SDS-PAGE. Anti apoLTP-I and anti apoLTP-III sera were easily raised while it was difficult to obtain anti apoLTP-II serum suggesting that apoLTP-II has low inherent immunogenicity. Immunoblot analysis of purified LTP with anti sera

raised against each apoprotein, demonstrated that each anti serum immunoreacted with only the apoprotein against which it was raised. There was no cross-reactivity between a particular apoprotein and anti serum against another apoprotein (Figure 4.1). These results indicate that LTP apoproteins are immunologically distinct polypeptides and that the antibodies are apoprotein-specific. The immunological uniqueness of the apoproteins confirms that apoLTP-II and -III are not proteolytic products of apoLTP-I. LTP is, therefore, unique among the plasma lipid transfer catalysts in its multimeric composition.

Since the apoprotein-specific antibodies were raised against denatured protein, it was necessary to demonstrate that they recognize holoLTP and to determine the apparent affinity of the antibodies against holoLTP. Polyclonal antibodies are a mixed population against many different epitopes, so it is not possible to determine a binding constant for each of the anti sera. LTP can not be resolved into native apoproteins since individual apoproteins are insoluble in buffer alone, making the determination of a titer for a particular apoprotein-specific antibody against the native apoprotein impossible. Thus, characterization of apoprotein-specific IgG was approached with enzyme-linked immunosorbent assays. Each of the apoprotein-specific antibodies were shown to recognize native LTP in antibody-capture ELISA experiments with different apparent affinities (Figure 4.2). Anti apoLTP-I IgG had the highest apparent affinity while anti apoLTP-II and -III had similar apparent affinities. This is consistent with the composition of the particle since apoLTP-I comprises approximately 60 % of the particle mass and would be expected to have a higher apparent affinity for an IgG population. Antigen-competition, antibody-capture ELISA demonstrated that the interaction of the apoprotein-specific IgG to LTP bound to the plate can be competed with LTP in solution,

further demonstrating that the apoprotein-specific antibodies recognize native LTP (Figure 4.3).

Numerous studies have demonstrated the catalytic ability of LTP (Ryan *et al.*, 1986a,b; Ryan *et al.*, 1988a,b; Ando *et al.*, 1990; Capurro and de Bianchi, 1990a; Hirayama and Chino, 1990; Ryan *et al.*, 1990c,d; Silver *et al.*, 1990; Singh *et al.*, 1992b; Takeuchi and Chino, 1993) but the apoprotein(s) responsible for lipid transfer have not been determined. Apoprotein-specific antibodies were utilized in a lipid transfer inhibition study to examine the catalytically important LTP apoprotein(s) (Figure 4.5). Anti apoLTP-II IgG effectively inhibited LTP activity in a turbidimetric assay of DAG transfer (Singh *et al.*, 1992a) at low concentrations of IgG while anti apoLTP-I and anti apoLTP-III IgG inhibited activity only at much higher IgG concentrations. The inhibition of transfer activity by anti apoLTP-II IgG becomes more important when viewed in context with the apparent affinities of the apoprotein-specific IgG for native LTP established by ELISA. Since anti apoLTP-I has a greater apparent affinity for native LTP than does anti apoLTP-II IgG, the results suggest that apoLTP-II is a catalytically important apoprotein. Inhibition of transfer activity by high levels of anti apoLTP-I and anti apoLTP-III IgG may be due to impaired access of LTP to substrate lipoproteins by the association of anti apoLTP-I or anti apoLTP-III IgG with the particle. The results presented here, however, do not entirely rule out a role for apoLTP-I or apoLTP-III in lipid transfer. Antibodies against epitopes of apoLTP-I or apoLTP-III involved in transfer may not be present in the antiserum prepared in this study or apoLTP-I or apoLTP-III may play roles in the interaction of LTP with tissues or lipoproteins *in vivo* (Prasad *et al.*, 1986a; Van Heusden and Law, 1989).

Apoprotein-specific antibodies were employed in immunoprecipitation experiments to examine the exposure of apoproteins on the surface of the particle and the stability of the complex. Antibodies directed against exposed apoproteins would be expected to immunoprecipitate the entire LTP complex while antibodies directed against buried apoproteins would be incapable of immunoprecipitating the particle. Results from the characterization of apoprotein-specific antibodies by ELISA suggest that each of the antibodies should immunoprecipitate the LTP complex. Radioiodination of LTP resulted in radiolabelling of apoLTP-I, apoLTP-II, and apoLTP-III indicating that all three are accessible to radioiodination. ApoLTP-III appeared to be radiolabelled to a greater extent than apoLTP-II suggesting that apoLTP-III has a larger number of tyrosine residues than does apoLTP-II. Immunoprecipitation of ^{125}I -LTP under native conditions demonstrated that apoLTP-I and apoLTP-II are exposed on the surface of the molecule since IgG purified from anti apoLTP-I and anti apoLTP-II sera were able to immunoprecipitate intact LTP. Anti apoLTP-III IgG were not able to immunoprecipitate ^{125}I -LTP indicating that the epitopes against which the apoLTP-III antisera were raised may be inaccessible. These results are in agreement with previous studies which demonstrated that apoLTP-III is protected from trypsin degradation while apoLTP-I and apoLTP-II are not (Ryan *et al.*, 1990b). Co-immunoprecipitation of apoLTP-I, apoLTP-II, apoLTP-III by anti apoLTP-I and anti apoLTP-II provides further evidence that LTP is, indeed, a multimeric complex consisting of three apoproteins. The immunoprecipitation results, however, seem to be in contradiction with the ELISA results since anti apoLTP-III IgG recognizes native LTP almost as well as anti apoLTP-II IgG. LTP activity inhibition by high concentrations of anti apoLTP-III also suggests apoLTP-III is partially exposed but this inhibition may

be due to steric hindrance of transfer rather than inhibition by IgG binding to epitopes involved in lipid transfer. These observations may indicate that the antibodies raised against apoLTP-III are not capable of immunoprecipitating apoLTP-III because immunoprecipitation requires a greater affinity to demonstrate a response than ELISA or steric inhibition of transfer. ApoLTP-III epitopes possibly exposed on the surface of LTP may not bind apoLTP-III with a high enough affinity to immunoprecipitate the entire complex.

Interesting results were obtained when the immunoprecipitation was carried out with a mild, non-ionic detergent included in the immune complex and purification of immune complex stages of the experiment. Under these conditions, anti apoLTP-I and anti apoLTP-II were both able to immunoprecipitate only apoLTP-I and apoLTP-II and not apoLTP-III which remained in the supernatant of the reaction. Anti apoLTP-III IgG, however, immunoprecipitated only apoLTP-III and cleared this apoprotein from the supernatant of the reaction mixtures. These results indicate that the integrity of the LTP complex is disrupted under these conditions. Mild, non-ionic detergents, such as Nonidet P-40, are not thought to denature proteins but do solubilize lipid (Neugebauer, 1990). This suggests that apoLTP-III may interact with lipid within the native particle. In the non-detergent containing immunoprecipitation experiments, anti apo-III IgG may not access apoLTP-III due to associated lipid and, therefore, would be unable to immunoprecipitate the complex. Additional evidence for the interaction of apoLTP-III with lipid and the protection of apoLTP-III against IgG binding by this lipid was obtained from ELISA characterization of the apoprotein-specific antibodies. When an ELISA was carried out for each of the apoprotein-specific antibodies under native conditions, the half maximal binding values for apoLTP-II and apoLTP-

III were 9 and 20 ng IgG/ μ l respectively. However, when non-ionic detergent was included in the ELISA, the values were 10 ng IgG/ μ l for both anti apoLTP-II and anti apoLTP-III IgG. These data suggest that anti apoLTP-III bound LTP more efficiently in the presence of detergent because apoLTP-III was more exposed than under native conditions. When non-ionic detergent was included in the immunoprecipitation and ELISA experiments, the interaction of apoLTP-III with lipid may have been disrupted, exposing apoLTP-III and allowing its dissociation from the particle. Previous studies demonstrated that SDS-treatment of LTP rendered apoLTP-III susceptible to trypsin degradation (Ryan *et al.*, 1990b) further suggesting apoLTP-III is protected from the aqueous environment by lipid. ApoLTP-III may not have been immunoprecipitated by anti apoLTP-I or anti apoLTP-II in the presence of NP-40 because it had dissociated from the complex. Support for this concept is provided by the observation that anti apoLTP-III was able to immunoprecipitate only apoLTP-III under these conditions. Thus, it appears that apoLTP-I and apoLTP-II are tightly associated while apoLTP-III can dissociate from the particle upon treatment with non-ionic detergent.

In summary, apoprotein-specific antibodies have been useful in examining structure and function of *M. sexta* LTP. These studies resulted in a number of important findings: LTP apoproteins are immunologically distinct polypeptides, apoLTP-II is involved in facilitating lipid transfer, apoLTP-I and apoLTP-II are exposed on the surface of the LTP complex, apoLTP-I and apoLTP-II form a more stable complex than that of apoLTP-III with the particle, and apoLTP-III appears to be associated with lipid.

Figure 4.1 Immunoblot of purified LTP with apoprotein-specific antibodies.

Purified LTP (0.2 μ g each lane) was run on a 4 - 12 % acrylamide gradient SDS-PAGE gel, transferred to PVDF membrane and probed with: lane 1, anti holoLTP (1 : 15 000 dilution of serum); lane 2, anti apoLTP-I (1 : 10 000); lane 3, anti apoLTP-II (1 : 5 000); lane 4, anti apoLTP-III (1 : 2 500); and non-immune (1 : 2 500) serum and detected with a biotinylated secondary antibody, a streptavidin linked horse-radish peroxidase and chemiluminescent horse-radish peroxidase substrate.

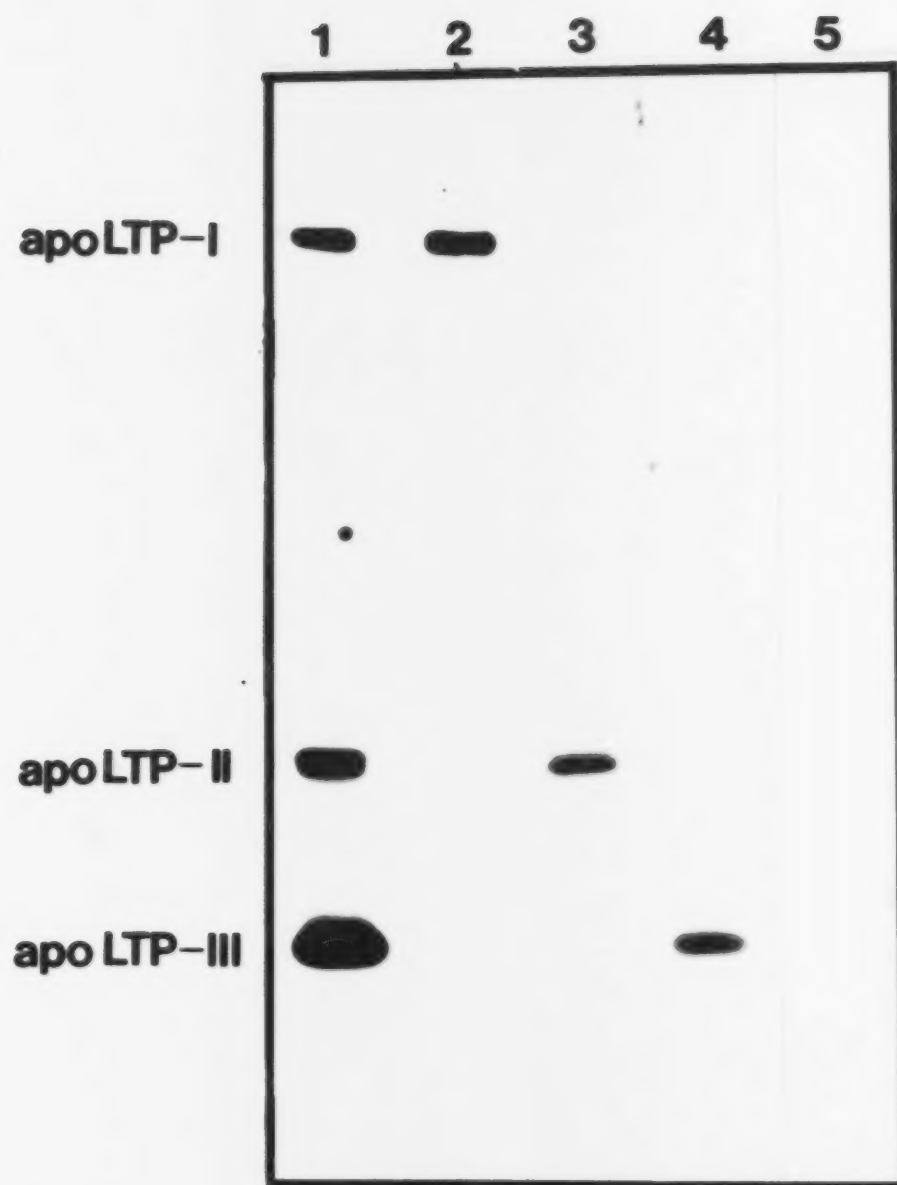


Figure 4.2 **Determination of apparent affinities of IgG preparations.**

Purified LTP (1 μ g) was bound to wells of Immulon 3 microtiter plates, excess protein binding sites were blocked, and primary antibodies (IgG; anti apoLTP-I; filled circles, anti apoLTP-II; open triangles, anti apoLTP-III; open circles, anti larval serum protein (control); filled triangles) in varying concentrations, as indicated, were allowed to immunoreact with the bound LTP. The extent of primary antibody binding was determined by detection with a horse-radish peroxidase-conjugated goat anti rabbit IgG and ABTS horse-radish peroxidase substrate. The absorbance at 405 nm is plotted versus the log of the IgG concentration. Error bars are the experimental deviation from triplicate determinations.

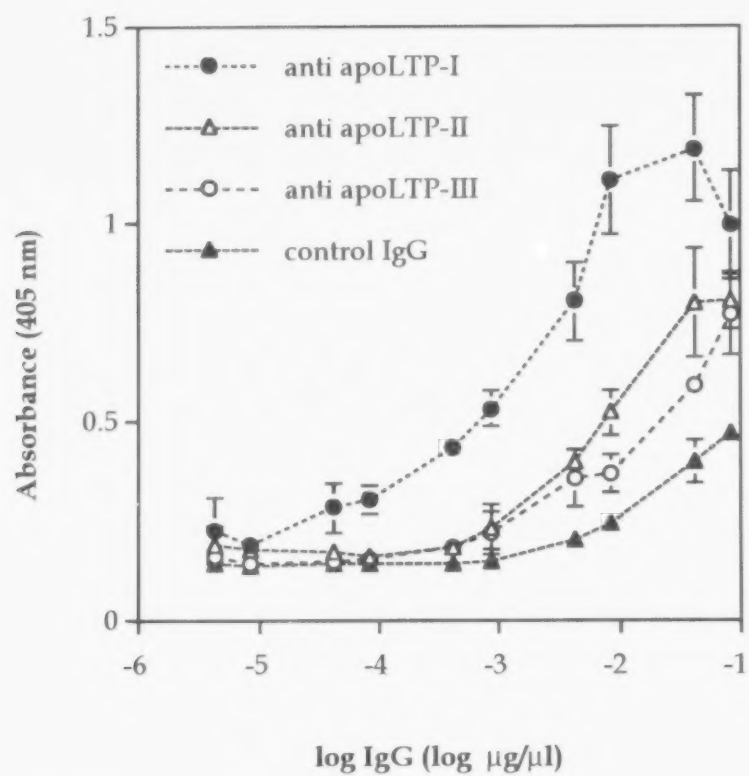


Figure 4.3 Competition ELISA with native LTP in solution.

Competition ELISA were carried out as described in Figure 2 except either buffer (no addition, open bars) or 100-fold excess soluble LTP (+ LTP, filled bars) were added with primary antibodies, anti larval serum protein (control), anti apoLTP-I, anti apoLTP-II, and anti apoLTP-III (IgG concentration at half maximal binding). Absorbance at 405 nm is plotted against the IgG tested. Error bars are the experimental deviation from triplicate determinations.

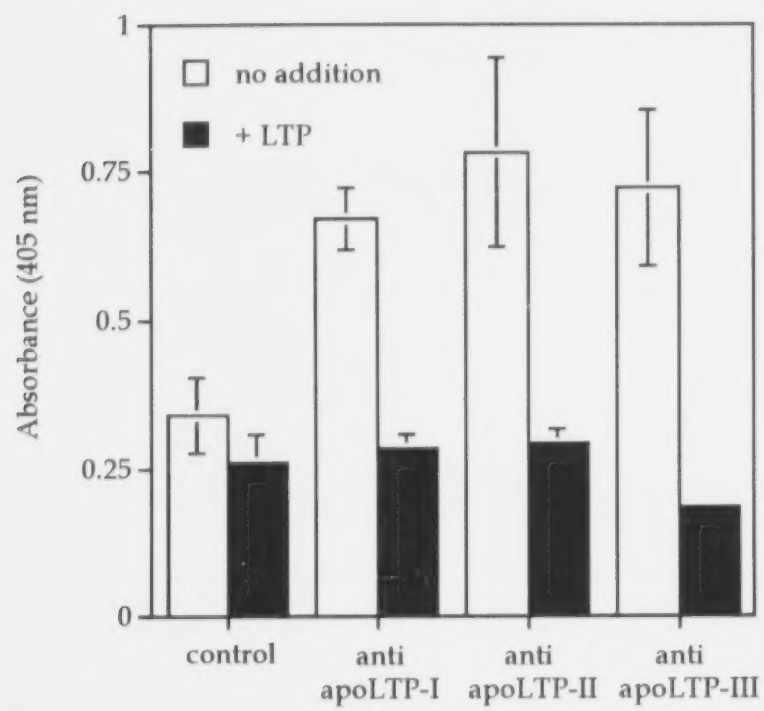


Figure 4.4 Characterization of apoprotein-specific antibodies in the presence of detergent.

Purified LTP (1 μ g) was bound to wells of Immulon 3 microtiter plates, excess protein binding sites were blocked with 0.5 % gelatin/0.05 % Tween-20/PBS, and primary antibodies (IgG; anti apoLTP-I; filled circles, anti apoLTP-II; open triangles, anti apoLTP-III; open circles, anti larval serum protein (control); filled triangles) in varying concentrations, as indicated, were allowed to immunoreact with the bound LTP. The extent of primary antibody binding was determined by detection with a horse-radish peroxidase-conjugated goat anti rabbit IgG and ABTS horse-radish peroxidase substrate. The absorbance at 405 nm is plotted here versus the log of the IgG concentration. Error bars are the experimental deviation from triplicate determinations.

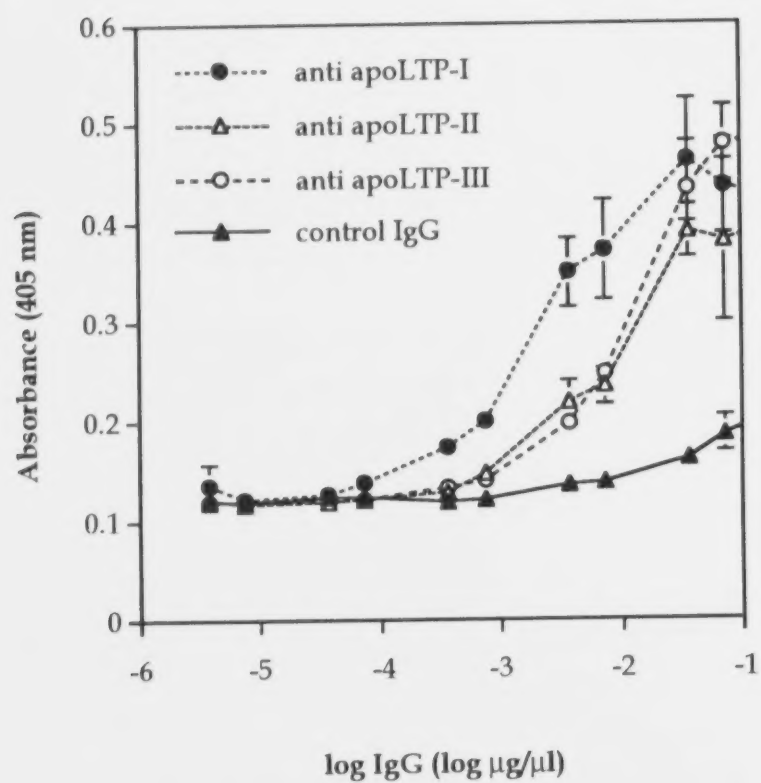


Figure 4.5 Effect of apoprotein-specific antibodies on LTP activity.

LTP (0.75 μ g) was incubated with increasing amounts of anti apoLTP-I (open squares), anti apoLTP-II (filled triangles), and anti apoLTP-III (filled circles) IgG to form immune complexes which were assayed for lipid transfer activity by a microtiter turbidimetric assay described in Experimental Procedures. Transfer activity was quantitated as the change in absorbance at 340 nm above control (absence of LTP) and expressed here as % maximal activity. The results presented are representative of experiments carried out three times. Error bars are the experimental deviation from triplicate determinations.

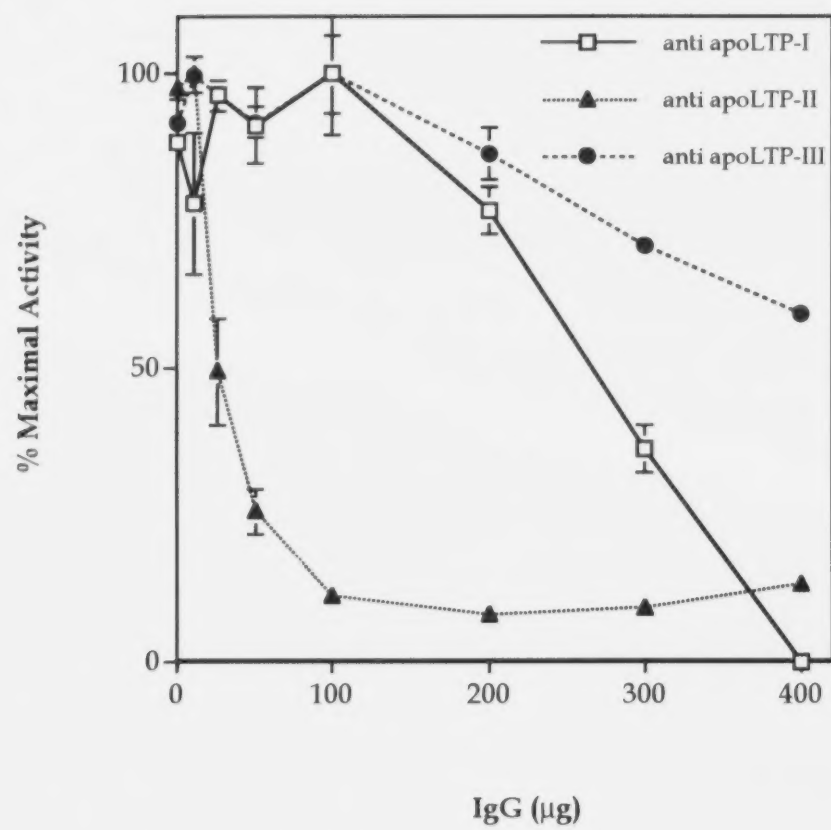


Figure 4.6 Double immunodiffusion analysis.

Eleven μ l of anti holoLTP (well A) and anti human apolipoprotein-B (well B) were placed in central wells and peripheral wells contained HDLp-W1 (13 μ g, well 1), human LDL (5 μ g, well 2), and LTP (10 μ g, well 3). Precipitin lines were stained after thorough washing with PBS and distilled H₂O with Coomassie Blue stain.

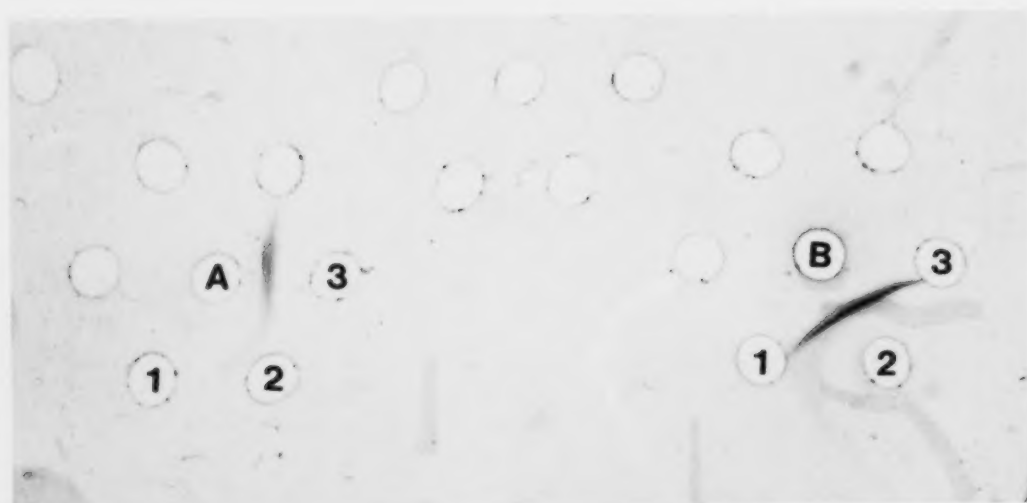


Figure 4.7 Immunoprecipitation of transfer activity from solution.

LTP (2 μ g) or PBS were preincubated with 2 μ l non-immune or anti holoLTP serum, immunoprecipitated with excess Protein-A Sepharose, and immune complexes were removed by centrifugation. 100 μ l aliquot of the supernatant was assayed for lipid transfer activity by incubation with 500 μ g HDLp-W1 protein and 100 μ g LDL protein and absorbance was read at 340 nm. Error bars are the experimental deviation from triplicate determinations.

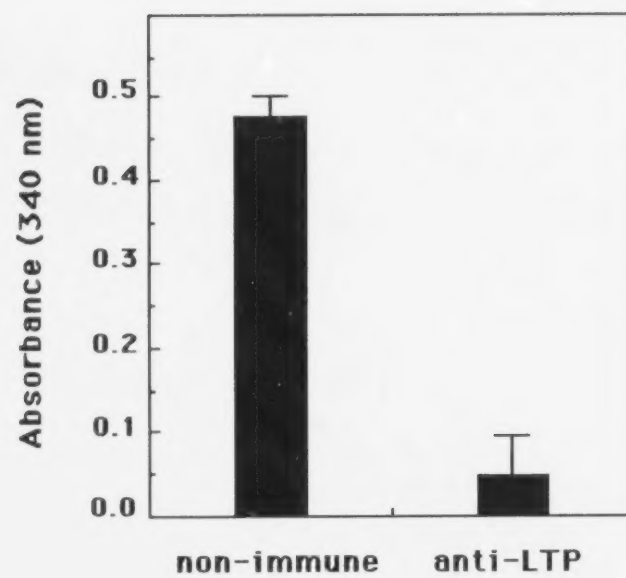


Figure 4.8 Immunoprecipitation under native conditions.

^{125}I -LTP (lane 1) was incubated under native conditions with non-immune (lane 2), anti holoLTP (lane 3), anti apoLTP-I (lane 4), anti apoLTP-II (lane 5), and anti apoLTP-III (lane 6) for 1 h at 4°C. Excess Protein A Sepharose was added and the sample was microfuged to pellet IgG - Protein A Sepharose complexes. The immunoprecipitated material (pellet) was solubilized and electrophoresed on a 4 - 12 % acrylamide gradient SDS-PAGE gel which was dried and autoradiographed.

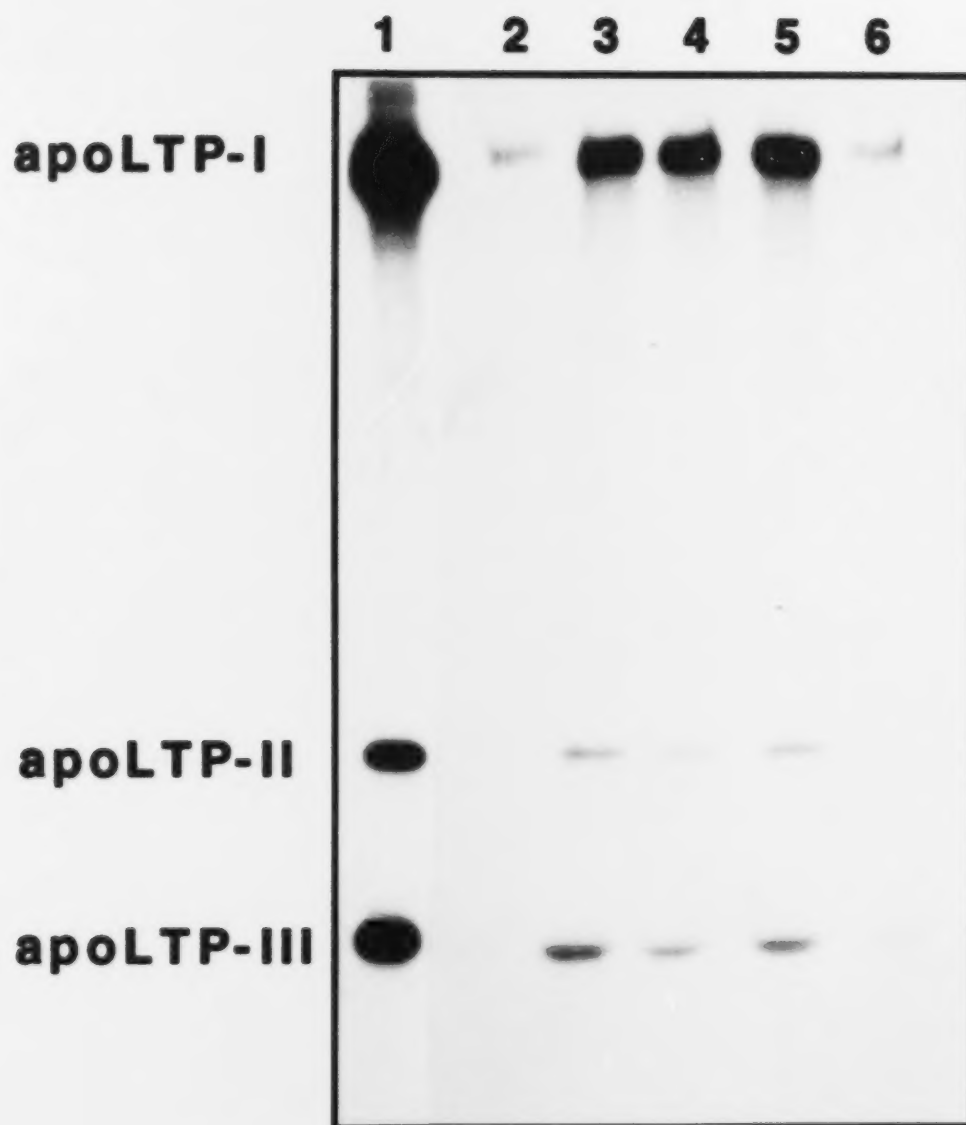
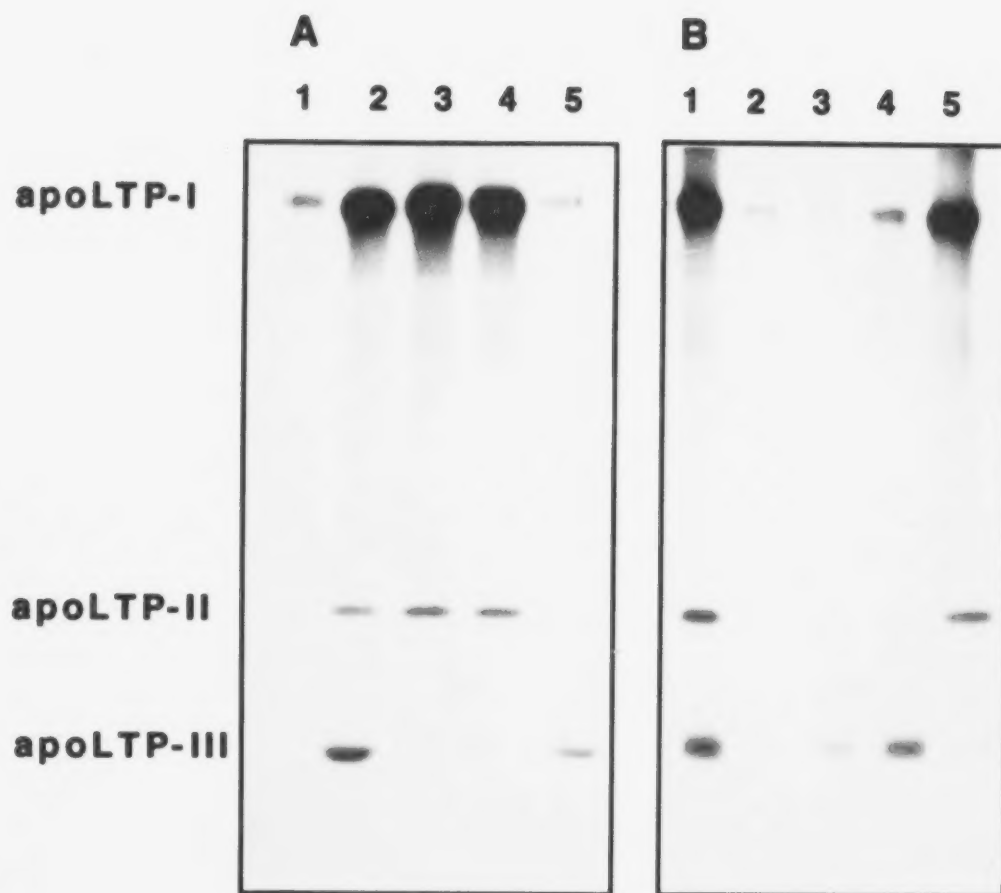


Figure 4.9 Immunoprecipitation in the presence of NP-40.

^{125}I -LTP was incubated in the presence of 0.5 % NP-40 with non-immune (lane 1), anti holoLTP (lane 2), anti apoLTP-I (lane 3), anti apoLTP-II (lane 4), and anti apoLTP-III (lane 5). Excess Protein A Sepharose was added and the sample was microfuged to pellet IgG - Protein A Sepharose complexes. Immunoprecipitated (pellets, Panel A) and non-immunoprecipitated material (supernatants, Panel B) were electrophoresed on a 4 - 12 % gradient SDS-PAGE gel which was dried and autoradiographed.



CHAPTER 5

FURTHER BIOCHEMICAL AND BIOPHYSICAL CHARACTERIZATION OF LIPID TRANSFER PARTICLE

5.1 Introduction.

Early studies of the structure of LTP revealed some fascinating characteristics including an extremely high molecular weight, the presence of three apoproteins and lipid, and a highly unusual morphology as evidenced by electron microscopy (Figure 1.3). These properties have led to many questions regarding the biochemical and physiological significance of the unique morphology, the spatial organization of the particle, and the role of the lipid and apoprotein components. Morphological studies of LTP utilized reagents such as trypsin, phospholipase A₂, and detergent to perturb LTP and electron microscopy and other biochemical techniques to examine their effects on the particle. Trypsin treatment under mild conditions resulted in the digestion of apoLTP-I and -II but not apoLTP-III. ApoLTP-III, however, was shown to possess trypsin-susceptible sites by digestion in the presence of SDS (Ryan *et al.*, 1990b). These results suggested that apoLTP-III or trypsin-susceptible sites of apoLTP-III are buried within the particle and protected from the aqueous environment. Phospholipase A₂ treatment of LTP was used to examine the location of the lipid component within the particle. While the enzyme was shown to hydrolyze LTP phospholipid to lysophospholipid and free fatty acid, electron microscopic examination of the treated particle did not reveal a morphological change (Ryan *et al.*, 1990b). The failure to induce a change in particle morphology upon phospholipase A₂ treatment may have been due to the association of lysophospholipid and free fatty acid products with the particle. Treatment of LTP with SDS caused perturbations in the head region of the particle suggesting that the lipid constituent of LTP resides in this area (Ryan *et al.*, 1990b). However, SDS treatment may have led to protein denaturation as well. The lipid component of LTP has been shown by a number

of studies to be important in lipid transfer (Ryan *et al.*, 1988c; Ryan *et al.*, 1990b). Chapter 4 described immunological experiments designed to examine structural and functional properties of LTP. Evidence was provided for the immunological distinctiveness of LTP apoproteins, the importance of apoLTP-II in lipid transfer activity, the inaccessibility of apoLTP-III to anti apoLTP-III in the intact particle, the relatively tight association of apoLTP-I and apoLTP-II, the interaction of apoLTP-III with lipid within the particle, and the dissociation of apoLTP-III from the particle upon treatment with a non-ionic detergent. This chapter will describe biochemical and biophysical studies which further characterize the structure and stability of the LTP complex, the nature of apoprotein association, and the isolation of LTP apoproteins.

The dissociation of apoLTP-III from the apoLTP-I/apoLTP-II complex in non-ionic detergent-containing immunoprecipitation experiments in Chapter 4 suggested that apoLTP-III is associated with lipid within the particle. Gel filtration chromatography of ^{125}I -LTP in the presence and absence of NP-40 was utilized to further examine the effects of the non-ionic detergent on apoprotein association. Electron microscopic visualization of LTP and ^{125}I -LTP in the presence and absence of NP-40 was employed to attempt to localize apoLTP-III and lipid within the LTP structure. The transfer activity of LTP and ^{125}I -LTP in the presence and absence of NP-40 was also examined.

Prior to the work presented in this dissertation, SDS-PAGE had been the only tool available for the separation of LTP apoproteins. The denaturation and dissociation of LTP in order to examine the nature of apoprotein interaction and, ultimately, obtain isolated apoproteins was, therefore, undertaken. Treatment with chaotropic agents has been utilized to achieve the denaturation of another lipid transfer catalyst, MTP, as well as the isolation of

one of its subunits (Wetterau *et al.*, 1990; Wetterau *et al.*, 1991b). Isolation of *M. sexta* apolipophorins, apoLp-I and apoLp-II, was also facilitated by denaturation with guanidine hydrochloride followed by gel filtration chromatography (Shapiro *et al.*, 1984; Kawooya *et al.*, 1989). In those studies, all of the lipophorin-associated lipid was recovered with isolated apoLp-I and lipophorin-like particles were reconstituted with phosphatidylcholine and isolated apoLp-I (Kawooya *et al.*, 1989). Circular dichroism spectroscopy and sedimentation velocity analytical ultracentrifugation were used to analyze LTP denaturation and the dissociation of apoproteins in the presence of the chaotropic agent, guanidine hydrochloride. The isolation of LTP apoproteins was attempted by guanidine hydrochloride gel filtration chromatography. ApoLTP-I and apoLTP-II were isolated by SDS denaturing gel filtration chromatography. Amino acid analysis and partial amino acid sequencing of LTP apoproteins is also discussed.

5.2 Results

5.2.1 NP-40 and native gel filtration chromatography of ^{125}I -LTP.

Immunoprecipitation of ^{125}I -LTP in the presence of NP-40 provided evidence that apoLTP-III can dissociate from the particle in the presence of a non-ionic, non-denaturing detergent. This was pursued in experiments aimed at isolating ^{125}I -apoLTP-III from the apoLTP-I/apoLTP-II complex. ^{125}I -LTP eluted from a Sepharose CL 6B gel filtration column with PBS as the elution buffer in one peak at fraction 80 (96 ml) as shown in Figure 5.1, panel A. A second peak of radioactivity was observed to elute at the total volume of the

column (183 ml, fraction 150). SDS-PAGE demonstrated that protein was not present in this peak indicating it corresponds to free ^{125}I . Phosphorimaging of a 4 - 12 % linear acrylamide gradient SDS-PAGE gel of selected fractions (Figure 5.2) demonstrated that the LTP particle remained intact and eluted from the column as a complex of apoLTP-I, -II, and -III. Gel filtration chromatography of ^{125}I -LTP preincubated with 1 % NP-40 using 1 % NP-40/PBS as the elution buffer with an identical column as above resulted in the elution of two peaks of radioactivity between fractions 71 and 89 (85 to 107 ml elution volume) as shown in Figure 5.1, panel B. Again, free ^{125}I eluted at the total volume of the column. Phosphorimaging of a 4 - 12 % linear acrylamide gradient SDS-PAGE gel of selected fractions from the 1 % NP-40 elution of ^{125}I -LTP revealed a partial dissociation of ^{125}I -apoLTP-III from the LTP complex (Figure 5.3). The fractions enriched in apoLTP-III eluted at the trailing end of the second peak of radioactivity. Densitometric scans by the phosphorimage analyzer of apoLTP-I, -II, and -III bands in fraction 79 of both the 1 % NP-40 and PBS elutions of ^{125}I -LTP revealed a densitometric ratio of apoLTP-I : apoLTP-II : apoLTP-III of 20 : 2.5 : 1 in the presence of NP-40 and 9.9 : 1.0 : 1.0 in the absence of NP-40. In fraction 91, the ratio was 14.7 : 3.4 : 9.3 in the presence of NP-40. These results demonstrate that apoLTP-III can dissociate from the particle and be resolved from the complex by gel filtration chromatography in the presence of 1 % NP-40.

5.2.2 Electron microscopy of ^{125}I -LTP and LTP in the presence and absence of NP-40.

Electron microscopy was employed to examine the morphological effects of incubation of LTP and ^{125}I -LTP with 0.1 % NP-40 (Figure 5.4 and 5.5). Comparison of micrographs of LTP and ^{125}I -LTP demonstrated that radioiodination has no gross morphological effects on LTP structure (Figure 5.4, panel A and Figure 5.5, panel A). Incubation of both LTP and ^{125}I -LTP with 0.1 % NP-40 resulted in significant perturbations in the morphology of the particle (Figure 5.4, panel B and Figure 5.5, panel B). These morphological changes seemed to be concentrated in the head region of the particle. Treatment with 0.1 % NP-40 also appeared to cause aggregation of the particles.

5.2.3 Effect of NP-40 on lipid transfer activity of LTP and ^{125}I -LTP.

The lipid transfer activity of LTP and ^{125}I -LTP preincubated in the presence and absence of 0.5 % NP-40 or treated with 0.5 % NP-40 immediately prior to the transfer assay was examined (Figure 5.6). LTP and ^{125}I -LTP, preincubated in the absence of NP-40, demonstrated a LTP or ^{125}I -LTP concentration dependence of transfer activity as shown by the linear increase in absorbance at 340 nm between 0.2 and 1 μg LTP (Figure 5.6, panel A) and 0.5 and 1 μg ^{125}I -LTP (Figure 5.6, panel B), both curves reached a plateau at 1.5 μg LTP. Preincubation of LTP or ^{125}I -LTP with 0.5 % NP-40 abolished lipid transfer activity throughout the range of LTP concentrations tested. As well, treatment of LTP or ^{125}I -LTP with 0.5 % NP-40 immediately prior to lipid

transfer assay abolished activity for all concentrations tested except 2.0 and 2.5 μg LTP.

5.2.4 Circular dichroism spectroscopy.

The denaturation of LTP by guanidine hydrochloride was examined by CD spectroscopy. LTP was treated with 3.0 and 6.0 M guanidine hydrochloride or not treated and the CD spectra measured. Native LTP has a high content of β -sheet secondary structure as demonstrated by the large trough at 218 nm (Figure 5.7). The loss of secondary structure upon treatment with 3.0 and 6.0 M guanidine hydrochloride is shown by the decrease in negative ellipticity at 218 nm (Figure 5.7). The guanidine hydrochloride concentration dependence of denaturation as monitored by the increase in ellipticity at 220 nm is shown in Figure 5.8 where the relative ellipticity at 220 nm is plotted as a function of guanidine hydrochloride concentration. This curve demonstrates that there was a loss of secondary structure in the LTP complex at concentrations as low as 0.7 M guanidine hydrochloride. At this concentration, there was a 10 % decrease in ellipticity. There was a broad transition from an ordered structure to a random structure between 0.7 and 6.0 M guanidine hydrochloride where the change in relative ellipticity reached a plateau. The absolute value of ellipticity, however, did not reach zero which would be indicative of a completely random structure. The midpoint for LTP denaturation was 2.3 M guanidine hydrochloride.

5.2.5 Sedimentation velocity experiments.

Analytical ultracentrifugation sedimentation velocity experiments indicated that LTP dissociates into smaller units and that this dissociation is dependent on guanidine hydrochloride concentration and ultracentrifugation time. Figure 5.9 are the schlieren patterns observed 19, 50, and 71 min (panels A, B, and C) into a sedimentation velocity experiment with native LTP and LTP treated with 3.0, 4.5, and 6.0 M guanidine hydrochloride. At 19 min into the analytical ultracentrifugation run (panel A), one peak was observed in the schlieren pattern of untreated LTP as well as LTP denatured by 3.0, 4.5, and 6.0 M guanidine hydrochloride. By 50 min into the experiment (panel B), native LTP had reached the bottom of the cell and LTP treated with 3.0 M guanidine hydrochloride appeared to maintain its multimeric structure as observed by the presence of only one peak in the schlieren pattern. LTP treated with 4.5 and 6.0 M guanidine hydrochloride, however, appeared to have dissociated into more than one species as demonstrated by the triplet of peaks in the 4.5 M guanidine hydrochloride and more than one peak in the 6 M guanidine hydrochloride schlieren patterns. At 71 min (panel C), LTP treated with 3.0 M guanidine hydrochloride had almost reached the bottom of the cell but had not yet dissociated into more than one species even though CD analysis demonstrated that considerable loss of LTP secondary structure occurs at this guanidine hydrochloride concentration. The schlieren pattern demonstrated that LTP treated with 6.0 M guanidine hydrochloride had dissociated into 3 species by this time point. The rate at which the LTP species reached the bottom of the cell depended upon the size of the species and the viscosity of the medium. Native LTP reached the bottom of the cell at a greater rate than guanidine hydrochloride-treated LTP due to the differences in viscosity of the media. Mr.

Leslie Hicks calculated the apparent $s_{20,w}$ for the leading peaks in the sedimentation velocity experiments using equations 1 and 2 given in the Materials and Methods chapter. Table 5.1 gives the apparent $s_{20,w}$ values for the fastest sedimenting species in each of the conditions. This value was in the range of 11.4 to 14.8 S for native LTP and LTP treated with 3.0, 4.5, and 6.0 M guanidine hydrochloride.

5.2.6 Guanidine hydrochloride/DTT denaturing gel filtration chromatography.

The dissociation of denatured LTP by sedimentation velocity analytical ultracentrifugation suggested that there was potential for the isolation of denatured apoproteins. Isolation of individual apoproteins was attempted by guanidine hydrochloride denaturing gel filtration chromatography. LTP was denatured with guanidine hydrochloride and DTT and subjected to gel filtration chromatography on a Sephadex G-150, 30 ml column with 6 M guanidine hydrochloride/1 mM DTT/50 mM Tris-base pH 8.0 as the elution buffer. This procedure was similar to that used in the isolation of apoLp-I and apoLp-II (Kawooya *et al.*, 1989). DTT was included in the denaturation step and elution buffer to prevent the formation of disulfide bridges between apoproteins. The protein eluted from the column in one peak as demonstrated by absorbance (280 nm) and SDS-PAGE analysis of the fraction containing the largest amount of protein revealed the presence of all three LTP apoproteins (data not shown).

5.2.7 SDS denaturing gel filtration chromatography.

The failure of guanidine hydrochloride denaturation to dissociate LTP apoproteins such that they could be isolated by gel filtration chromatography led to attempts to resolve the apoproteins by SDS denaturing gel filtration chromatography. LTP was treated with 2 % SDS and subjected to gel filtration chromatography with a Sepharose CL 6B column with 2 % SDS/PBS as the elution buffer. This allowed the isolation of apoLTP-I after one chromatography step. ApoLTP-II was isolated after fractions enriched in apoLTP-II were pooled and subjected to further SDS gel filtration chromatography. Figure 5.10 is a SDS-PAGE gel demonstrating the isolation of apoLTP-I and apoLTP-II.

5.2.8 Amino acid analysis of LTP apoproteins by hydrolysis from PVDF membrane.

LTP apoproteins were separated by SDS-PAGE, transferred to PVDF membrane and subjected to hydrolysis from the membrane by 6 M HCl at high temperature by the Alberta Peptide Institute. This procedure did not allow the determination of Trp or Cys residues. Treatment of PVDF-bound apoproteins under conditions which allow determination of Trp and Cys did not result in hydrolysis. The amino acid compositions of apoLTP-I, apoLTP-II, and apoLTP-III are given in Table 5.2. Calculation of the prevalence of hydrophobic amino acids (Val, Ile, Leu, Ala, Phe, Met, Pro) revealed that all three apoproteins have high contents of nonpolar residues, apoLTP-I; 37 %, apoLTP-II; 44 %, and apoLTP-III; 46 %. When Kyte/Doolittle hydrophathy indices (Kyte and Doolittle, 1982) were used to assign hydrophobicity to amino acid residues, that

is, a residue was considered to be hydrophobic when its hydropathy index was greater than zero (Ala, Val, Leu, Ile, Phe, Met, Cys), the nonpolar amino acid contents of the apoproteins (not including Cys) were apoLTP-I; 33 %, apoLTP-II; 39 %, and apoLTP-III; 40 %. When the same estimations are made for the hydrophobic residue content calculated from the average occurrence of amino acids in proteins (Klapper, 1977), the former method indicated 40 % of the residues in an average protein are hydrophobic while the use of Kyte/Doolittle hydropathy indices reveals 33 % of an average protein are hydrophobic residues. These comparisons demonstrate that apoLTP-II and apoLTP-III are more hydrophobic than the average protein and that apoLTP-III is slightly more hydrophobic than apoLTP-II. ApoLTP-I is a relatively hydrophilic polypeptide.

5.2.9 Partial amino acid sequence from LTP apoprotein trypsin fragments.

Trypsin fragments were generated as previously described (Ryan *et al.*, 1990b). These fragments were transferred to PVDF membrane and microsequenced by Mr. Mike Carpenter. Both apoLTP-I and apoLTP-III amino termini were blocked to Edman degradation. Partial sequence were obtained for apoLTP-II amino terminus and a fragment of each apoLTP-I and apoLTP-II as given below.

apoLTP-I fragment 1: SLPSTQXA

apoLTP-II fragment 1: AG/WSVINKRSNLLHXXTSYIXF

apoLTP-II amino terminus: SNSLKPPYIC/PO₄-SXPPYXSP

(one letter codes for amino acids, G/W: either G or W, C/PO₄-S: either C or phosphoserine)

5.3 Discussion

The work described in this chapter was designed to further characterize the organization of the LTP complex and the nature of the interaction of LTP apoproteins and lipid. The isolation of LTP apoproteins was also pursued.

Studies of the effect of NP-40 treatment of ¹²⁵I-LTP were extended with gel filtration chromatography, electron microscopic morphological studies, and lipid transfer assays in the presence of NP-40. Gel filtration chromatography in the presence of NP-40 allowed the resolution of apoLTP-III from the LTP complex (Figure 5.3). This indicates that apoLTP-III is associated with lipid within the particle. This lipid may have a role in the interaction of apoLTP-III with the apoLTP-I/apoLTP-II complex observed in the immunoprecipitation experiments reported in Chapter 4. The immunoprecipitation results presented in Chapter 4 demonstrated that in the presence of NP-40, ¹²⁵I-apoLTP-III completely dissociated from the LTP complex since anti apoLTP-I and anti apoLTP-II IgG did not immunoprecipitate apoLTP-III. In the absence of NP-40, however, the entire LTP complex was immunoprecipitated by these antibodies. Dissociation of ¹²⁵I-apoLTP-III by gel filtration chromatography was not complete. These results suggest that apoLTP-III dissociation is in equilibrium with its association with LTP. The binding of antibodies in the immunoprecipitation experiment may have driven the equilibrium in favour

of dissociation. In the gel filtration experiment, apoLTP-III may have had the opportunity to dissociate and reassociate with the complex resulting in the observation of partial dissociation. Visualization of LTP and ^{125}I -LTP in the presence and absence of 0.1 % NP-40 by electron microscopy revealed significant perturbation of the head region when LTP and ^{125}I -LTP were exposed to 0.1 % NP-40 (Figure 5.4 and 5.5). Taken together with the failure of anti apoLTP-III IgG to immunoprecipitate native LTP, the dissociation of apoLTP-III from apoLTP-I/apoLTP-II complex upon immunoprecipitation in the presence of NP-40, and the resolution of apoLTP-III from the particle by NP-40 gel filtration chromatography, the disruption in the head region of LTP upon NP-40 treatment observed by electron microscopy suggests that apoLTP-III associates with lipid and is perhaps covered by this lipid in the head region of the particle. The greater hydrophobicity of apoLTP-III relative to the other apoproteins, as determined by amino acid analysis, supports the hypothesis that this apoprotein is sequestered from the aqueous medium.

The effect of NP-40 on lipid transfer activity of LTP and ^{125}I -LTP was examined by the turbidimetric transfer assay to determine if apoLTP-III association is required for LTP activity (Figure 5.6). ^{125}I -LTP was shown to have comparable lipid transfer activity with native LTP demonstrating that tyrosine modification does not effect lipid transfer and suggesting that tyrosine residues may not be involved in lipid transfer. Incubation of LTP and ^{125}I -LTP in the presence of 0.5 % NP-40 abolished lipid transfer activity at all LTP and ^{125}I -LTP concentrations as assayed by the turbidimetric assay. Transfer activity in the presence of 0.5 % NP-40 would have indicated that apoLTP-III association with the complex is not required for lipid transfer. It is unclear if the observed lack of transfer activity was due to NP-40-induced dissociation of

apoLTP-III or lipid, NP-40 effects on substrate lipoproteins in the assay, or a direct inhibition of transfer by NP-40 which was distinct from lipid solubilization. The slight increase in sample turbidity at greater than 2 μ g LTP in the presence of NP-40 without preincubation and the observation that the absorbance of substrate lipoproteins is the same in the absence or presence of NP-40 tends to rule out NP-40 effects on substrate lipoproteins as the only factor in NP-40 inhibition of transfer activity.

The denaturation and possible dissociation of LTP into constituent components was examined by two biophysical techniques, circular dichroism spectroscopy and analytical ultracentrifugation. Previous CD studies allowed the calculation of the relative contributions of secondary structure elements to LTP structure. LTP was shown to contain 6 % α -helix, 49 % β -sheet, and 35 % random coil by CONTIN analysis (Ryan *et al.*, 1990a). In this study, CD spectroscopy demonstrated a loss in LTP secondary structure upon treatment with 3.0 and 6.0 M guanidine hydrochloride (Figure 5.7). The dependence of LTP denaturation on guanidine hydrochloride concentration was also examined by CD spectroscopy. LTP denaturation commenced at low guanidine hydrochloride concentrations (0.7 M) and proceeded slowly until 6.0 M guanidine hydrochloride when complete denaturation was achieved as evidenced by a plateau in the relative ellipticity plot (Figure 5.8). The broad transition demonstrated by the guanidine hydrochloride denaturation plot revealed that LTP denaturation is a complex, multistep process. This is indicative of stable folding intermediates and may suggest that there are several independently folded domains within the particle which denature independent of each other but at equivalent guanidine hydrochloride

concentrations. A complex denaturation curve would be expected for an entity as complex as LTP.

CD spectroscopy revealed that LTP is partially denatured by low concentrations of guanidine hydrochloride and that denaturation is complete at 6.0 M guanidine hydrochloride. The possibility of the dissociation of LTP into constituent apoproteins upon guanidine hydrochloride denaturation was examined by analytical ultracentrifugation. Native LTP and LTP treated with 3.0, 4.5, and 6.0 M guanidine hydrochloride were subjected to sedimentation velocity experiments during which photographs of the schlieren patterns were taken at three time points (Figure 5.9). Native LTP did not dissociate and the $s_{20,w}$ calculated was similar to that previously reported (Ryan *et al.*, 1990a). LTP treated with 3.0 M guanidine hydrochloride also did not appear to dissociate even though significant denaturation was shown to occur at this guanidine hydrochloride concentration by CD. The $s_{20,w}$ calculated for LTP under this condition was similar to that of native LTP. Treatment of LTP with either 4.5 or 6.0 M guanidine hydrochloride resulted in dissociation into at least three species. The $s_{20,w}$ of the species corresponding to the leading peak in each of these schlieren patterns, however, was similar to that of native LTP suggesting that dissociation was not complete. Evidence for the lack of dissociation of LTP apoproteins upon guanidine hydrochloride treatment was obtained from the failure of guanidine hydrochloride denaturing gel filtration chromatography to resolve LTP apoproteins. Dissociation of mammalian MTP into its 88 000 and 55 000 Da subunits occurs at concentrations as low as 1.2 M guanidine hydrochloride (Wetterau *et al.*, 1991a). The results obtained here indicate that the LTP complex is a great deal more stable than the MTP complex. The lipid component of LTP may confer stability to the complex in spite of significant

denaturation. Kawooya *et al.* (Kawooya *et al.*, 1989) demonstrated that lipid can remain bound to an apolipoprotein upon denaturation by guanidine hydrochloride.

Sequential denaturing gel filtration chromatography in the presence of 2 % SDS allowed the isolation of apoLTP-I and -II in large quantities. SDS appears to denature LTP and induce the dissociation of the apoproteins. This is probably due to the ability of SDS to both solubilize lipid and denature protein secondary structure by binding through hydrophobic interactions.

The work presented in this chapter represents further characterization of insect lipid transfer particle by a variety of biochemical and biophysical techniques. These results have advanced our understanding of the structure of LTP and will be the basis for further investigations into the structure and function of LTP. In this chapter the association of apoLTP-III with lipid in the head region of the particle has been suggested. As well, the nature of the interaction of LTP apoproteins and their resilience to dissociation upon denaturation by guanidine hydrochloride and the possible role lipid plays in this has been discussed. Finally, LTP apoproteins have been isolated from each other for the first time in solution.

Figure 5.1 Gel filtration chromatograph of ^{125}I -LTP eluted in the presence and absence of NP-40.

^{125}I -LTP (specific activity = 5.4×10^4 cpm/ μg ; 0.3 mg) incubated with either PBS (panel A) or 1 % NP-40 (panel B) was subjected to gel filtration chromatography with an 183 ml Sepharose CL 6B column with PBS or 1 % NP-40/PBS, respectively, as elution buffer. Aliquots (50 μl) of the 1.2 ml fractions were counted on a gamma counter and the cpm per aliquot is plotted here against the fraction number.

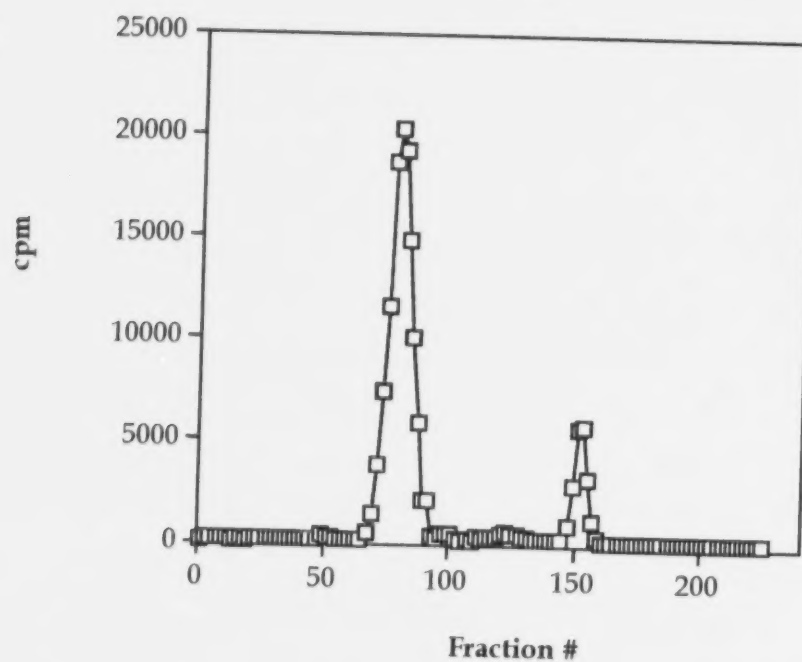
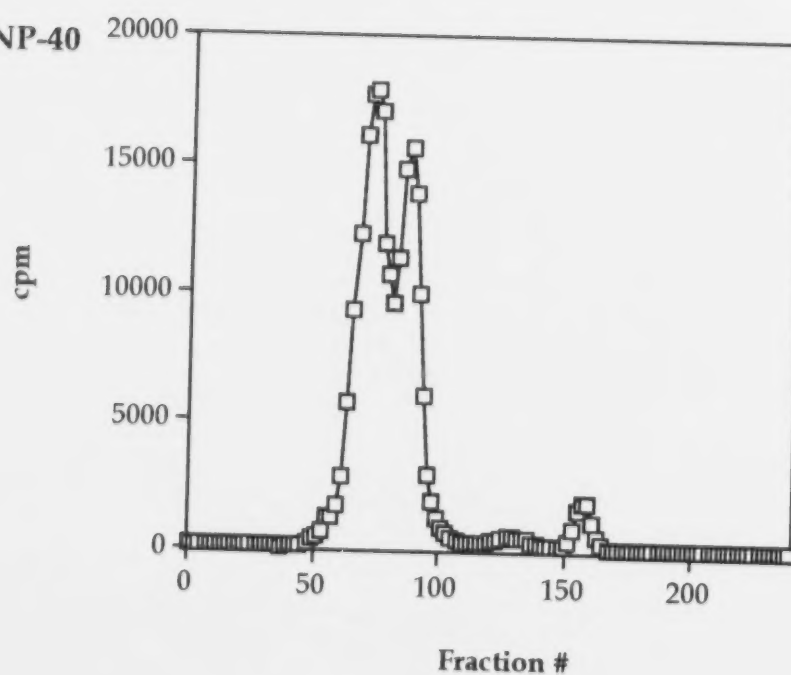
A PBS**B 1 % NP-40**

Figure 5.2 Phosphorimage of SDS-PAGE of fractions from gel filtration chromatography of ^{125}I -LTP.

^{125}I -LTP (specific activity = 5.4×10^4 cpm/ μg ; 0.3 mg) incubated with PBS was subjected to gel filtration chromatography (Sephacrose CL 6B column matrix, 183 ml) with PBS as the elution buffer. Aliquots (50 μl) of selected eluted fractions containing radioactivity (every fourth fraction from 51 to 111) were electrophoresed on a 4 - 12 % linear acrylamide gradient SDS-PAGE gel. The gel was dried and detected by phosphorimaging. The leftmost lane is control ^{125}I -LTP (10 μl).

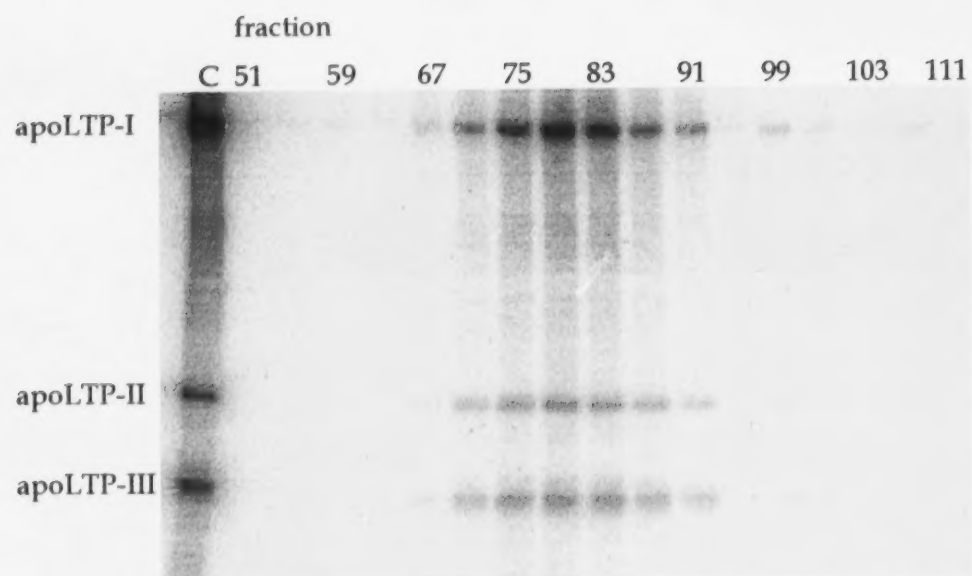


Figure 5.3 Phosphorimage of SDS-PAGE of fractions from gel filtration chromatography of ^{125}I -LTP in the presence of NP-40.

^{125}I -LTP (specific activity = 5.4×10^4 cpm/ μg ; 0.3 mg) incubated with 1 % NP-40 was subjected to gel filtration chromatography (Sephacrose CL 6B column matrix, 183 mL) with 1 % NP-40/PBS as the elution buffer. Aliquots (50 μl) of selected eluted fractions containing radioactivity (every fourth fraction from 51 to 111) were electrophoresed on a 4 - 12 % linear acrylamide gradient SDS-PAGE gel. The gel was dried and detected by phosphorimaging. The leftmost lane is control ^{125}I -LTP (10 μl).

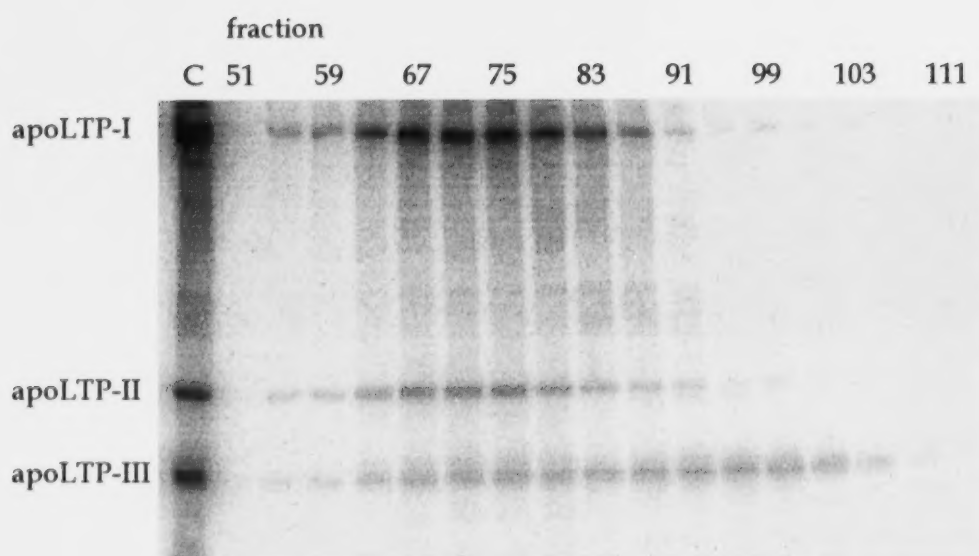


Figure 5.4 Electron micrographs of LTP in the presence and absence of NP-40.

LTP was treated with PBS (panel A) or 0.1 % NP-40 (panel B) and subjected to electron microscopy with 2 % sodium phosphotungstate negative staining.

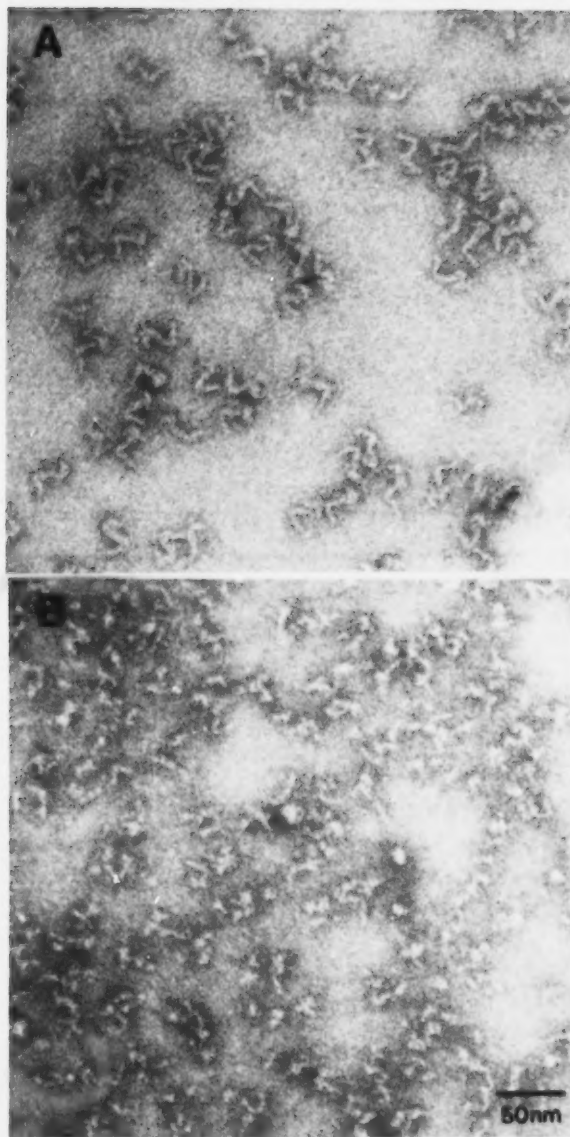


Figure 5.5 Electron micrographs of ^{125}I -LTP in the presence and absence of NP-40.

LTP was treated with PBS (panel A) or 0.1 % NP-40 (panel B) and subjected to electron microscopy with 2 % sodium phosphotungstate negative staining.

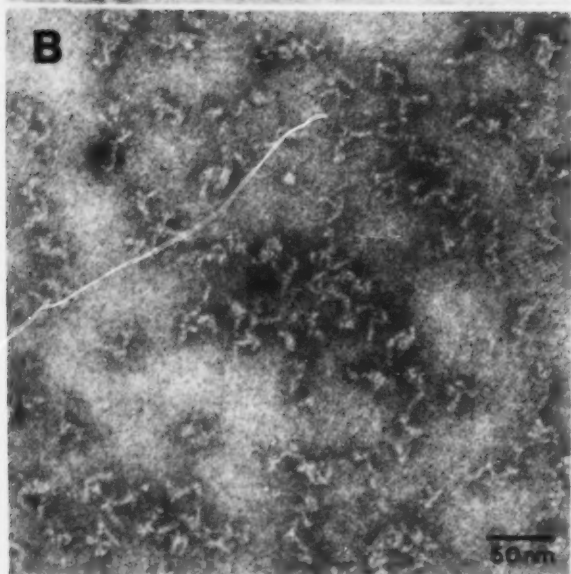
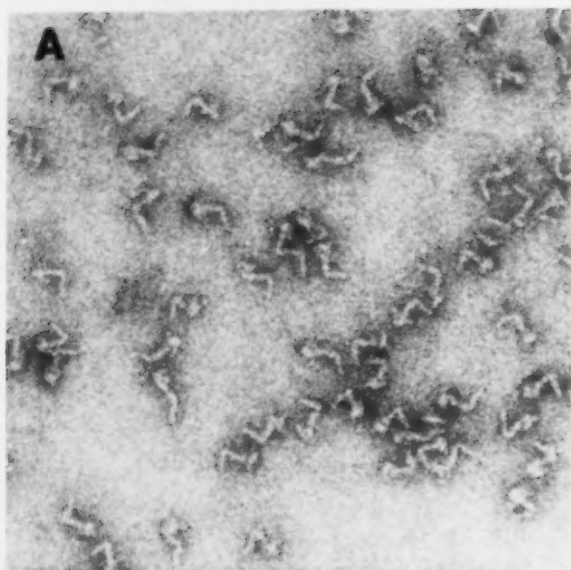


Figure 5.6 Turbidimetric lipid transfer assay of LTP and ^{125}I -LTP in the presence and absence of NP-40.

LTP (panel A) and ^{125}I -LTP (panel B) in amounts from 0.2 to 2.5 μg were assayed for lipid transfer activity by the turbidimetric assay after preincubation for 1 h with PBS (open squares) or 0.5 % NP-40 (open triangles) or immediately after addition of 0.5 % NP-40 (open circles) in microtiter plate wells. HDLp-W1 (500 μg protein) and LDL (100 μg protein) were added and incubated for 45 min at 37°C. Transfer activity was determined by the absorbance at 340 nm, plotted here versus amount of LTP or ^{125}I -LTP. The results shown are the mean of triplicate determinations and the error bars are the experimental error.

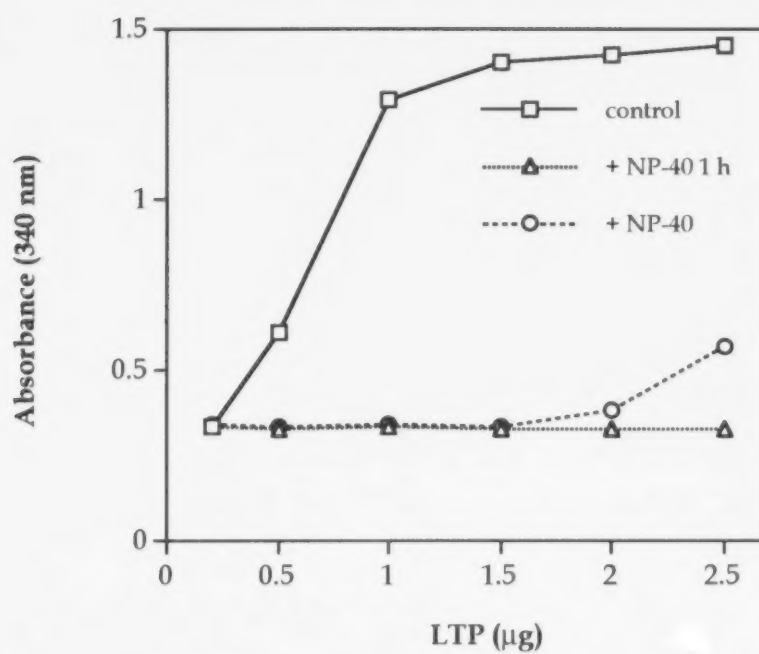
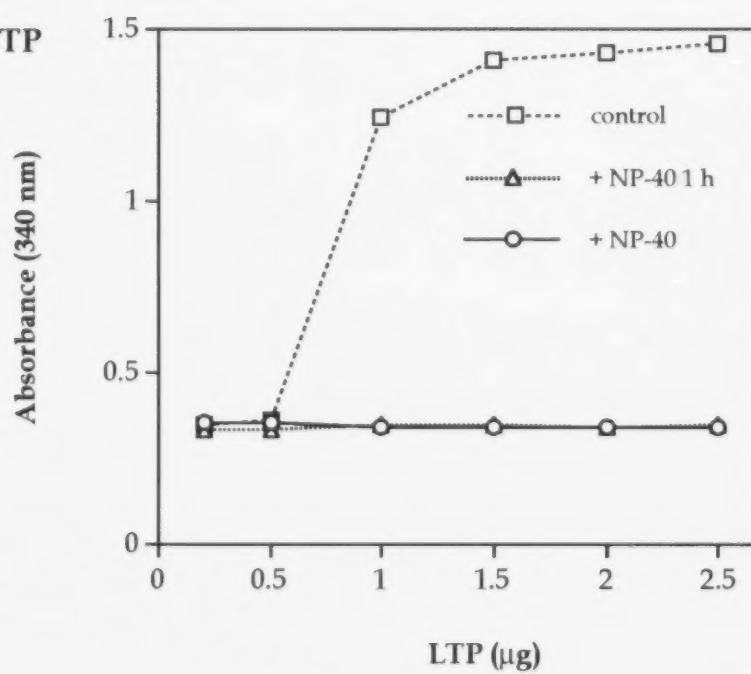
A LTP**B ^{125}I -LTP**

Figure 5.7 Circular dichroic spectra of LTP and LTP treated with 3.0 and 6.0 M guanidine hydrochloride.

LTP treated with 3.0 and 6.0 M guanidine hydrochloride and native LTP were subjected to circular dichroic spectroscopy. The molar ellipticity is plotted here against wavelength for native LTP (solid line), and LTP treated with 3.0 M (dotted line) and 6.0 M (dashed line) guanidine hydrochloride.

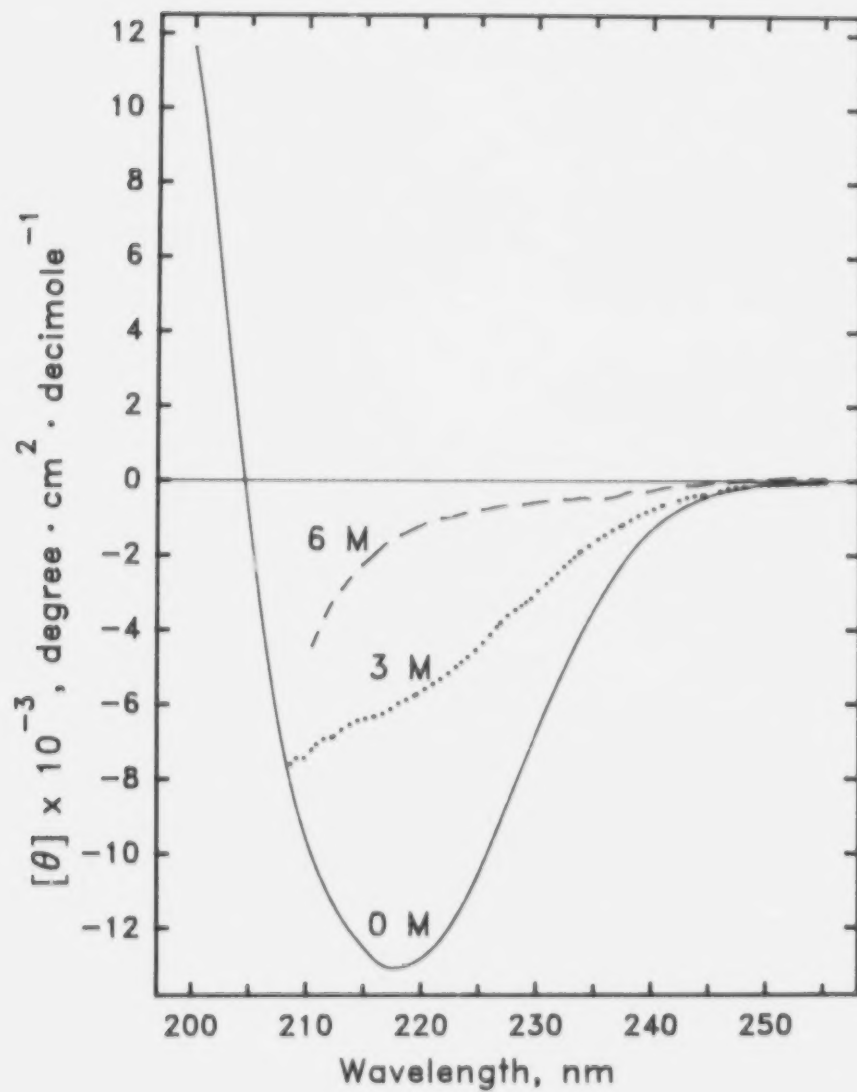


Figure 5.8 Guanidine hydrochloride concentration dependence for the denaturation curve of LTP.

LTP was treated with guanidine hydrochloride in concentrations ranging from 0 to 7.3 M and the cd spectra obtained. The extent of denaturation is plotted here as relative ellipticity at 220 nm (native LTP was set at 100 %) versus guanidine hydrochloride concentration.

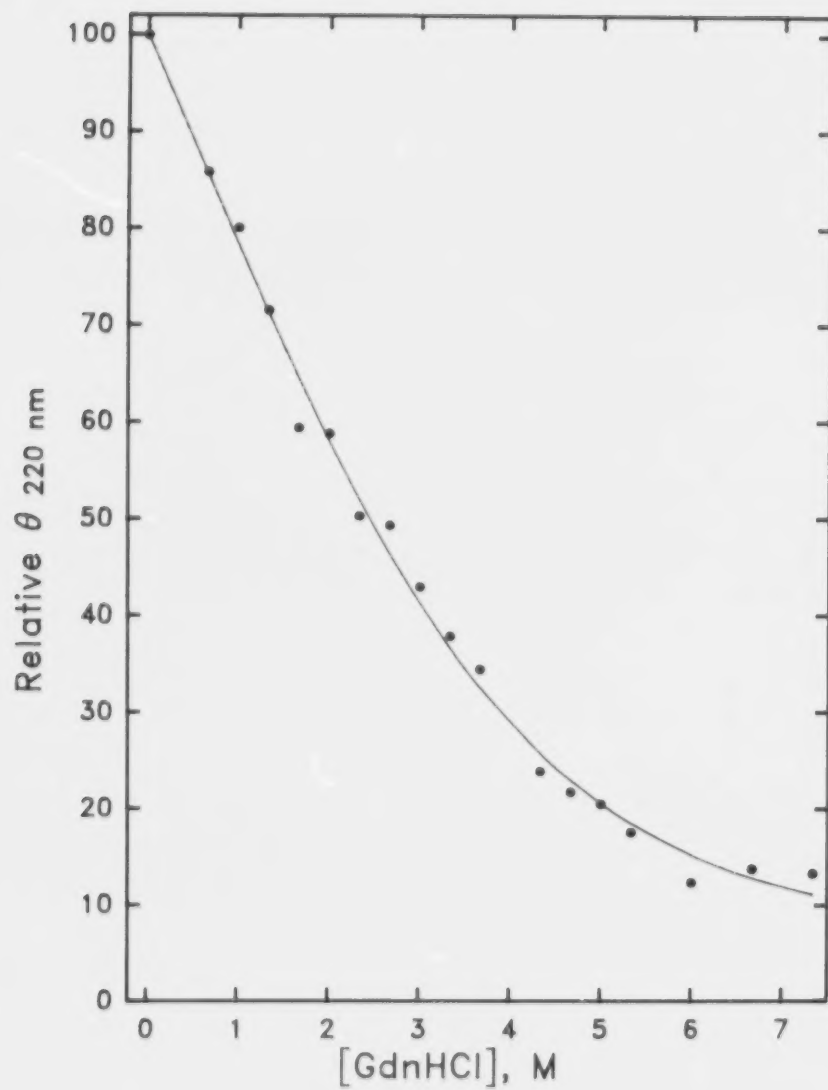
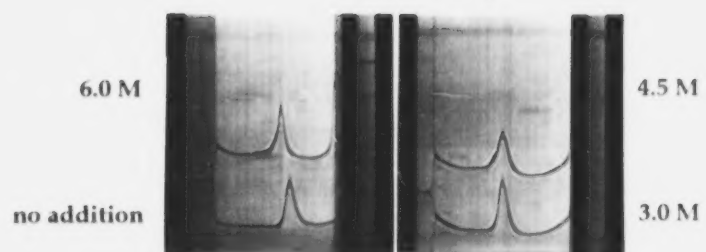


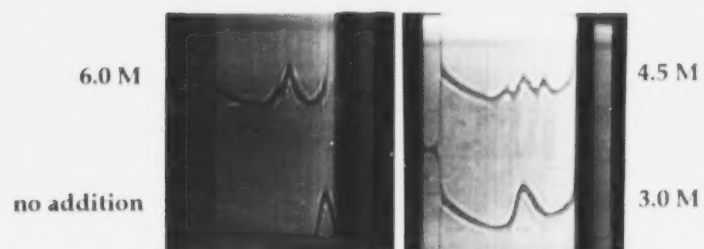
Figure 5.9 Schlieren photographs from sedimentation velocity experiments of native LTP and LTP treated with 3.0, 4.5, 6.0 M guanidine hydrochloride.

LTP treated with 3.0, 4.5, and 6.0 M guanidine hydrochloride and native LTP were subjected to sedimentation velocity analytical ultracentrifugation and photographs of schlieren patterns were taken at 19 (panel A), 50 (panel B), and 71 min (panel C) are shown here.

A



B



C

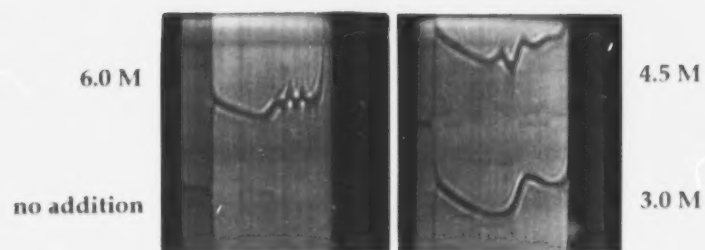


Table 5.1 Apparent $s_{20,w}$ of leading peaks of native LTP and LTP treated with 3.0, 4.5, 6.0 M guanidine hydrochloride.

Schlieren photographs of sedimentation velocity analytical ultracentrifugation experiments with LTP treated with 3.0, 4.5, and 6.0 M guanidine hydrochloride and native LTP were used to calculate the apparent $s_{20,w}$ of the fastest sedimenting species.

Sample	Apparent $s_{20,w}$
LTP no addition	13.9
LTP + 3.0 M Gdn HCl	11.4
LTP + 4.5 M Gdn HCl	12.9
LTP + 6.0 M Gdn HCl	14.8

Figure 5.10 SDS-PAGE of isolated LTP apoproteins.

LTP (2.8 mg) was subjected to SDS denaturing gel filtration chromatography (Sephacrose CL 6B matrix, 183 ml) to isolate LTP apoproteins. Isolated LTP apoproteins were electrophoresed on a 4 - 12 % linear acrylamide gradient SDS-PAGE gel shown here. Lane 1; high molecular weight markers, (myosin, 200 000; β -galactosidase, 116 000; phosphorylase *b*, 93 000, albumin; 67 000, and ovalbumin, 45 000), lane 2; LTP (50 μ g), lane 3; apoLTP-I, and lane 4; apoLTP-II.

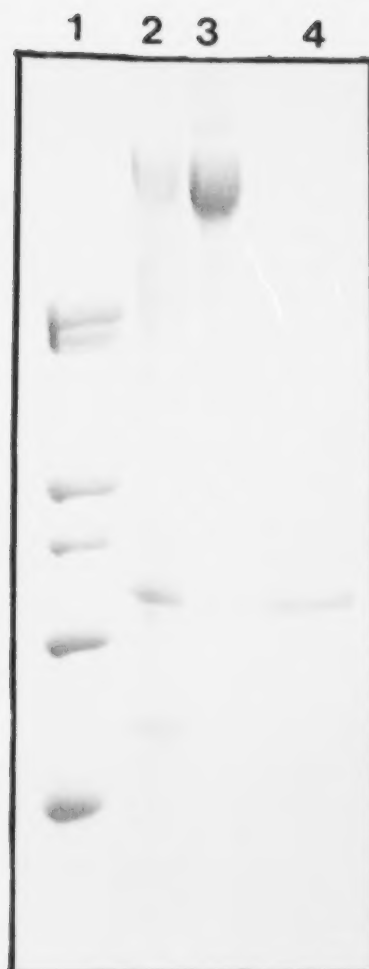


Table 5.2 Amino acid composition of apoLTP-I, apoLTP-II, and apoLTP-III.

LTP (658 μ g) was electrophoresed on a 4 - 12 % linear acrylamide gradient SDS-PAGE, transferred to PVDF, and the apoproteins were hydrolyzed from the PVDF membrane and analyzed. The absolute amount of each amino acid and the mol % for each apoprotein is given here. Trp and Cys residues are not included in these results.

Amino Acid	apoLTP-I		apoLTP-II		apoLTP-III	
	nmol	mol %*	nmol	mol %*	nmol	mol %*
Asx	7.6095	14.6	13.1917	11.6	11.1412	11.6
Thr	2.9261	5.62	6.2155	5.5	5.2814	5.5
Ser	2.8038	5.39	6.3191	5.6	3.6808	3.8
Glx	4.7439	9.11	14.5751	12.8	14.6164	15.2
Gly	7.8789	15.1	7.2351	6.4	8.1639	8.5
Ala	3.6514	7.02	6.3374	5.6	6.3552	6.6
Val	3.1378	6.03	9.2949	8.2	8.4531	8.8
Met	1.2497	2.40	2.9649	2.6	1.8733	2.0
Ile	1.9059	3.66	7.2889	6.4	7.6749	8.0
Leu	5.1426	9.88	12.2427	10.8	8.2802	8.6
Tyr	1.8994	3.65	4.2026	3.7	2.6588	2.8
Phe	2.3077	4.43	6.4517	5.7	5.3582	5.6
His	0.9455	1.82	2.1014	1.9	0.2173	2.3
Lys	2.4611	4.73	4.9406	4.4	4.2450	4.4
Arg	1.3883	2.67	4.3960	3.9	2.3031	2.4
Pro	1.9970	3.84	5.7720	5.1	5.7230	6.0

* mol% values were calculated without Trp and Cys.

CHAPTER 6

GENERAL DISCUSSION

The purpose of the work presented in this dissertation was to characterize structural and functional properties of *M. sexta* lipid transfer particle. LTP is a high molecular weight complex of three apoproteins and lipid found in insect hemolymph which has the unique ability to catalyze the net vectorial transfer and exchange of lipids between lipoprotein substrates and from tissues to lipoproteins (for reviews see: Ryan, 1990; Blacklock and Ryan, 1993; Blacklock and Ryan, 1994). Physiologically, LTP is thought to be involved in the transfer of dietary lipids from the midgut to lipoproteins (Prasad *et al.*, 1986a), in the mobilization of fat body lipid stores for flight energy (Van Heusden and Law, 1989), and in the interconversion of hemolymph lipophorins (Ryan *et al.*, 1986a). Very little is known about the mechanism used by LTP to accomplish lipid transfer or the physiological and biochemical significance of the unique properties of LTP. Many of the characteristics of LTP; its size, possession of three apoproteins and lipid, unique morphology and lipid transfer ability make it an interesting model for studies of lipid transfer, protein-lipid, and protein-protein interactions. This thesis has extended our knowledge of LTP and how it functions as a lipid transfer catalyst.

Studies described in this thesis utilize a number of approaches in examining aspects of the structure and function of LTP. The mechanism of action of LTP was examined by a novel solid-phase substrate system which allowed the physical separation of donor and acceptor lipoproteins. Conclusive observations of the ability of LTP to utilize a carrier-mediated mechanism in the transfer of DAG between lipoproteins were obtained from this study. At the same time, the contribution of transfer via a ternary complex mechanism was shown to be insignificant in the system used. This work was

the first clear demonstration of a plasma lipid transfer catalyst utilizing a carrier-mediated mechanism of action in lipid transfer.

The structure and function of LTP was probed with polyclonal antibodies raised against each of the three apoproteins of LTP. These antibodies were used to examine the immunological relationship of LTP apoproteins, the role of the apoproteins in lipid transfer, and the spatial organization and stability of the LTP complex. Important results were obtained which establish the immunological uniqueness of the apoproteins and identify apoLTP-II as a catalytically important apoprotein. The exposure of apoLTP-I and apoLTP-II on the surface of the particle, the relative stability of interactions between apoLTP-I and apoLTP-II, and the protection of apoLTP-III from the aqueous environment through its interaction with lipid were demonstrated.

Structural studies were extended with experimentation aimed at further characterizing the stability of the LTP complex with the objective of isolating individual apoproteins. LTP was denatured by detergents as well as the chaotropic salt, guanidine hydrochloride. Electron microscopic studies of non-ionic detergent-treated LTP provided further support for the hypothesis that the lipid constituent of LTP resides the head region of the particle and, along with immunoprecipitation and gel filtration chromatography results, suggest that apoLTP-III is associated with this lipid. Characterization of LTP denatured with guanidine hydrochloride by CD spectroscopy and sedimentation velocity analytical ultracentrifugation showed that the denaturation of LTP is a complex, multistep process which results in the partial dissociation of the particle. Unfortunately, attempts to isolate guanidine hydrochloride denatured LTP apoproteins were unsuccessful. SDS-denaturing gel filtration chromatography, however, did allow the isolation of each of the LTP apoproteins.

A carrier mechanism of action might be expected when the physiological functions of LTP are taken into account (Prasad *et al.*, 1986b; Ryan *et al.*, 1986a; Van Heusden and Law, 1989). For example, in the case of the interconversion of lipophorins already present in the hemolymph compartment, LTP could be envisioned to acquire lipid from a donor lipophorin, travel through the hemolymph, and donate it to an acceptor lipophorin. This transfer must be carried out quickly since at any one stage of development only one subspecies of lipophorin is present in the hemolymph (Ryan *et al.*, 1986a). Van Heusden and Law (1989) demonstrated that LTP is required for the loading of DAG from the fat body into lipophorin. Lipophorin then ferries the lipid to the flight muscle where it is hydrolyzed and used for energy. Figure 1.1 depicts the players in the mobilization response and shows LTP in a ternary complex with the fat body and lipophorin. There is no physiological evidence for this since the interaction of LTP with fat body and lipophorin has not been investigated. A fat body lipophorin receptor has been identified (Tsuchida and Wells, 1990) but is thought to play a role in the transfer of dietary lipid from lipophorin for storage. LTP appears not to be involved in this since antibodies raised against LTP did not inhibit the transfer of radiolabelled DAG from lipophorin to the fat body but did inhibit transfer of DAG out of the fat body to lipophorin (Van Heusden and Law, 1989). This implies that the fat body lipophorin receptor is not involved in the transfer of lipid out of the fat body into lipophorin and may be interpreted as suggesting that the loading of lipid into lipophorin does not require direct interaction of lipophorin with the fat body. This may evoke a carrier-mediated mechanism of transfer for LTP in this physiological situation. A ternary complex of LTP with lipophorin and fat body in the absence of a receptor binding of lipophorin may not be necessary. A revised model for the transfer of DAG from fat body to lipophorin is presented in Figure 6.1.

The question of whether LTP uses a carrier-mediated or ternary complex mechanism in lipid transfer in the insect also requires consideration of the interaction of LTP with the fat body. All of the assay systems used to examine LTP activity, including the system utilized in the mechanism of transfer studies, are *in vitro* systems in which transfer occurs by relatively long-range movement through the aqueous medium. This is not necessarily the situation *in vivo*. The carrier-mediated mechanism demonstrated in these studies may be important *in vivo* but a more local carrier mechanism can be envisioned at the fat body if LTP is anchored to the tissue. This interaction may be through the tail of LTP and the hinge may allow the movement of the lipid-containing head region back and forth from fat body to lipophorin (Figure 6.1).

The immunological studies and the examination of the effect of non-ionic detergent on LTP may provide clues as to the role of each apoprotein in transfer within the animal. Inhibition of LTP transfer activity by apoLTP-II-specific antibodies demonstrated that apoLTP-II is important in lipid transfer. Perhaps apoLTP-II is situated just below the head region of the particle poised for transfer. The lipid-containing domain of the particle has been demonstrated by SDS (Ryan *et al.*, 1990b) and NP-40 treatment with electron microscopic visualization to be in the head region of the particle. ApoLTP-III appears to be associated with this lipid within the LTP complex. Anti apoLTP-III IgG did not inhibit lipid transfer activity at low concentrations where the inhibition effect would be expected to be mechanistic rather than through steric hindrance. If the head region contains lipid which is involved in the lipid transfer activity of LTP and apoLTP-III is associated with this region, a role for apoLTP-III in lipid transfer may be expected. Inhibition studies described in this thesis may not have detected a role for apoLTP-III in lipid transfer because the apoprotein was

protected from antibody binding by associated lipid. Alternatively, antibodies against epitopes involved in lipid transfer may have been absent from the antiserum preparation used. Unreported observations in which the relative amount of apoLTP-III compared to apoLTP-I and apoLTP-II seems to vary among preparations may indicate that apoLTP-III is not stably associated with the particle and perhaps dissociates and reassociates with LTP depending on lipid content. The relatively low lipid content of LTP has, thus far, not allowed the isolation of lipid-depleted and lipid-loaded species of LTP. Comparison of the apoprotein content of such species may provide clues as to the role of apoLTP-III. The size of apoLTP-I and the inhibition of transfer activity only at high IgG concentrations suggest this apoprotein serves as a structural frame for the particle. It may be expected that the hinge is part of this apoprotein suggesting an important role for apoLTP-I in the physiological situation.

The results described in this dissertation provide the basis for experimentation to further elucidate the structure and function of *M. sexta* LTP and other lipid transfer systems. Experiments that may be useful in this are proposed here.

Since both ternary complex and carrier-mediated transfer mechanisms require interaction between LTP and lipoproteins, further studies should be conducted to examine the nature of this interaction. LTP and substrate lipid particles may interact through hydrophobic and electrostatic forces. It may be possible to distinguish between these interactions by employing substrates covalently linked to a Sepharose matrix as in the mechanism studies to examine the elution of LTP through this column under different ionic strength and pH conditions.

The circulation system utilized in this study could be applied to the mechanism of transfer of other lipids between lipoproteins or the mechanism of action of lipid transfer proteins of other species. For example, the system could be used to examine the mechanism of action of the plasma CETP. There may be difficulty in that one of the substrates of CETP, HDL, does not possess integral apoproteins. The dissociation of apoproteins from HDL as lipid is exchanged may compromise the value of the system. Sparks *et al.* (1987) developed an assay to determine the CE transfer activity of plasma. [^3H]CE-HDL covalently bound to CNBr-activated-Sepharose was incubated with plasma and transfer activity was determined without the need for isolation of plasma lipoproteins by counting plasma supernant after pelleting of [^3H]CE-HDL-CNBr-activated-Sepharose. This assay was not compromised by the possible dissociation of apolipoproteins upon transfer suggesting that the circulation system may be useful in examining the mechanism of action of CETP. The mechanism of action of intracellular lipid transfer proteins such as MTP could also be examined with this system although substrates which can be bound to a Sepharose matrix would need to be developed. One possibility for this would be lipid vesicles with a small percentage of lipid analogues which have groups that would allow covalent linkage to the matrix. MTP has also been shown to facilitate the transfer of TAG and CE from LDL to HDL with a preference for TAG (Wetterau and Zilversmit, 1985). These lipoprotein substrates may prove useful in examining the mechanism of action of both CETP and MTP.

The transfer of lipids between particles may also be examined with the circulation system described in Chapter 3. Lipids can transfer between lipoproteins through aqueous diffusion, particle collision, or protein-facilitated

transfer. Solid-phase lipid particles would be useful in distinguishing between aqueous diffusion and particle collision transfer mechanisms. Lipid particles containing radiolabelled or otherwise labelled lipids could be covalently bound to Sepharose matrices, buffer circulated between columns, and transfer analyzed as in the experiments described in this thesis. If particle collision is required for the transfer of a particular lipid species, movement of that lipid would not be expected when both donor and acceptor particles are bound but would be expected if one of the particles were circulated.

The apoprotein-specific antibodies generated in Chapter 4 have many potential uses in the further study of LTP. The spatial organization of LTP may be investigated by allowing the apoprotein-specific antibodies to bind LTP and then utilize electron microscopy to visualize the IgG bound particles. ELISA and immunoprecipitation experiments demonstrated that anti apoLTP-I and anti apoLTP-II IgG bind native LTP. Electron microscopy should permit the visualization of these IgG molecules bound to LTP, allowing the localization of LTP apoproteins within the particle. Anti apoLTP-III would probably not bind native LTP under these conditions since it was not able to immunoprecipitate the LTP complex. Electron microscopy has been used in the past to examine the binding of subunit- or fragment-specific IgG to a multimeric protein. Immunocomplexes of fatty acid synthetase and antibodies against fragments of one its subunits were viewed by rotary shadowing electron microscopy. This revealed antibody-decorated fatty acid synthetase molecules with specific IgG attachment sites. These studies allowed the construction of a three dimensional model for the structure of fatty acid synthetase (Kitamoto *et al.*, 1988). Antibodies have been especially useful in the characterization of structure and function of prokaryotic and eukaryotic ribosomes. Ribosomal subunits and

rRNA have been localized within the extremely complex particles by immunoelectron microscopy, as well (Tischendorf *et al.*, 1974; Tischendorf *et al.*, 1975; Kastner *et al.*, 1981; Luhrmann *et al.*, 1981; Winkelmann *et al.*, 1982). Similar studies could be carried out with LTP to determine the apoprotein localization in the head, hinge, or tail region of the particle. This would provide invaluable information of the mechanism of lipid transfer and the possible role of the apoproteins within the particle.

The mechanisms of LTP assembly and recruitment of constituent lipid should be investigated. *M. sexta* apoLp-I and apoLp-II have been shown to originate from a common precursor, proapolipophorin (Weers *et al.*, 1993). Similarly, the subunits of mosquito vitellogenin are the products of proteolytic cleavage of a larger precursor (Bose and Raikhel, 1988; Dhadialla and Raikhel, 1990). This approach to lipoprotein biosynthesis by the fat body appears to ensure the simultaneous secretion of two apoproteins, one having a high molecular weight and, the other, a small molecular weight, and maintenance of the 1 : 1 ratio of these subunits within the lipid-protein complexes. An analogous, but more complex, problem must be encountered by the fat body during the biosynthesis of LTP since this requires the simultaneous secretion of three apoproteins, each in two copies. Apoprotein-specific antibodies would be extremely useful in investigations into the biosynthesis of LTP. Immunoprecipitation of fat body homogenates with these antibodies should reveal if LTP apoproteins also arise from a common precursor. The origin of LTP-associated lipid should also be investigated.

Other uses for apoprotein specific antibodies include examination of the immunorelationships between LTP from different insect species as well and between LTP and lipid transfer proteins from higher organisms. They would

prove very useful in the screening of an expression cDNA library for the cloning and sequencing of LTP apoproteins. The amino acid sequence obtained from the trypsin fragments in Chapter 5 would be useful in the development of probes for screening of a cDNA library or primers for polymerase chain reaction cloning of the apoproteins of LTP. Degenerate primers for polymerase chain reaction cloning of apoLTP-II have been designed from the limited amino acid sequence information available and preliminary experiments have been conducted in the laboratory. Amino acid sequence of additional fragments of LTP apoproteins, especially those generated by CNBr digestion, would be appropriate for the design of probes and primers. Preliminary CNBr digestion of holoLTP has been carried out and a number of fragments were produced. Separation of these fragments and identification of originating apoproteins is required. This could be accomplished by separating the fragments by high performance liquid chromatography and identification of the apoproteins from which the fragments arose by ELISA with apoprotein-specific antibodies as established in Chapter 4. The isolation of LTP apoproteins by SDS-denaturing gel filtration chromatography provides large amounts of apoproteins which may serve as substrates for CNBr or proteolytic digestion to produce fragments suitable for amino acid sequencing.

NMR and ESR (electron spin resonance) spectroscopy, differential scanning calorimetry, and small angle x-ray scattering have been used to examine the organization of lipid within lipid-protein complexes such as insect lipophorins, human lipoproteins, and *Xenopus laevis* vitellogenin (Banaszak and Seelig, 1982; Birrell *et al.*, 1982; Katagiri *et al.*, 1985; Hamilton and Morrisett, 1986; Katagiri *et al.*, 1987; Fenske *et al.*, 1990; Katagiri *et al.*, 1991; Wang *et al.*, 1992). Similar studies could be carried out with LTP which is of comparable

size with lipophorins and similar lipid content with the lipovitellin/phosvitin complex (vitellogenin). Studies in which a series of spin-labelled lipids were introduced into vitellogenin and analysis of the ESR spectra as well as NMR experiments allowed the characterization of the organization of the lipid in the complex (14 % by weight). The lipid of vitellogenin was shown to occur in a lipid-rich region or pool which has a bilayer-like component (Banaszak and Seelig, 1982; Birrell *et al.*, 1982). Examination of LTP by these methods would provide information as to the nature of the organization of lipid within the particle.

Lipid transport in insects serves as a useful, simplified model for lipid transport in general. Information gleaned from this system is invaluable to our understanding of lipoprotein metabolism in higher organisms, including mammals. Mammalian lipoprotein metabolism is significantly more complex than insect lipoprotein metabolism. While insects have lipophorin subspecies based on the same basic matrix particle consisting of apoLp-I and apoLp-II and a small amount of phospholipid, there are distinct groups of structurally different mammalian lipoproteins; the very low density lipoproteins, the intermediate and low density lipoproteins, and the high density lipoproteins.

The very low and low density lipoproteins are similar in structure to insect lipophorins. These particles are essentially spherical having a hydrophobic core and an amphipathic surface region comprised of phospholipid, cholesterol, and apoproteins. Both of these classes of lipoproteins have one integral apolipoprotein, apo B, which establishes the structural stability of the particles. The very low density lipoproteins, chylomicrons, produced by enterocytes, and VLDL, originating in the liver, are responsible for the delivery of diet-derived or *de novo* synthesized TAG to

peripheral tissues. LDL is a CE-rich lipoprotein and acts to transport CE between tissues. HDL particles are comprised of CE in the core of the particle and surface PL and apolipoproteins which freely exchange with other lipoproteins within circulation. HDL is involved in the transport of cholesterol through the plasma compartment, especially in reverse cholesterol transport from peripheral tissue to the liver. Plasma lipoprotein metabolism depends on the action of lecithin : cholesterol acyltransferase and CETP to redistribute lipids among lipoproteins. Lecithin : cholesterol acyltransferase catalyzes the esterification of lipoprotein free cholesterol with a fatty acid group derived from surface phosphatidylcholine. CETP mediates the exchange of TAG and CE between apo B-containing lipoproteins and HDL. (Davis, 1991; Fielding and Fielding, 1991; Schneider, 1991)

CETP has been purified, cloned, and the cDNA and genomic sequence determined. This hydrophobic glycoprotein has a molecular weight in the range of 66 000 to 74 000 as determined by SDS-PAGE and demonstrates a micro-heterogeneity, apparently based on varying glycosylation states (Tall, 1993). CETP is capable of transferring CE, TAG, retinyl ester, and PL between lipoproteins and artificial substrate particles. It is responsible for all of the plasma neutral lipid transfer activity but only part of the phospholipid transfer activity. A plasma PLTP has been described with remarkably similar properties to CETP. PLTP is a protein of M_r 70 000 which transfers PL but not neutral lipids between lipoproteins (Tollefson *et al.*, 1988). A physiological role for PLTP has not been clearly determined, but since PL-enriched HDL seems to act as a better substrate for CETP, it has been suggested that these two transfer proteins act in synergy (Tall, 1993).

Of the intracellular lipid transfer catalysts, two classes have emerged: the monomeric, cytosolic lipid transfer proteins such as the phospholipid transfer proteins which transport lipid through the cell (for example, phosphatidylinositol transfer protein, nonspecific phospholipid transfer proteins, and phosphatidylcholine transfer proteins) and have molecular masses of 8 000 to 35 000 Da and the microsomal triacylglycerol transfer protein complex (MTP).

MTP was originally isolated as a noncovalent heterodimer of two polypeptides (88 000 Da and 58 000 Da) from bovine liver (Wetterau and Zilversmit, 1985; Wetterau *et al.*, 1990; Wetterau *et al.*, 1991a) and has been shown to be present in the lumen of microsomes isolated from liver and intestinal mucosa (Wetterau and Zilversmit, 1986). The smaller subunit of MTP was found to be the multi-functional protein disulfide isomerase (PDI) (Wetterau *et al.*, 1990) and the larger subunit is thought to be the catalytically active component (Wetterau *et al.*, 1990). PDI, which, independently, has no TAG transfer ability, is required for lipid transfer ability and solubility of the larger subunit (Wetterau *et al.*, 1991b). MTP catalyzes the transport of TAG, CE, and PC between membranes (Wetterau and Zilversmit, 1984) and has been implicated in the transfer of TAG into apo B-containing lipoproteins during lipoprotein assembly. Patients with abetalipoproteinemia, which is characterized by an absence of apo B-containing lipoproteins in the plasma and low plasma concentrations of TAG and CE, have been shown to be deficient in the 88 000 Da subunit of MTP and MTP TAG transfer activity in intestinal biopsy samples (Wetterau *et al.*, 1992). Cloning of the cDNA of the 88 000 Da subunit allowed the analysis of the gene in abetalipoproteinemic patients (Sharp *et al.*, 1993). Distinct gene defects were found in the MTP gene of two

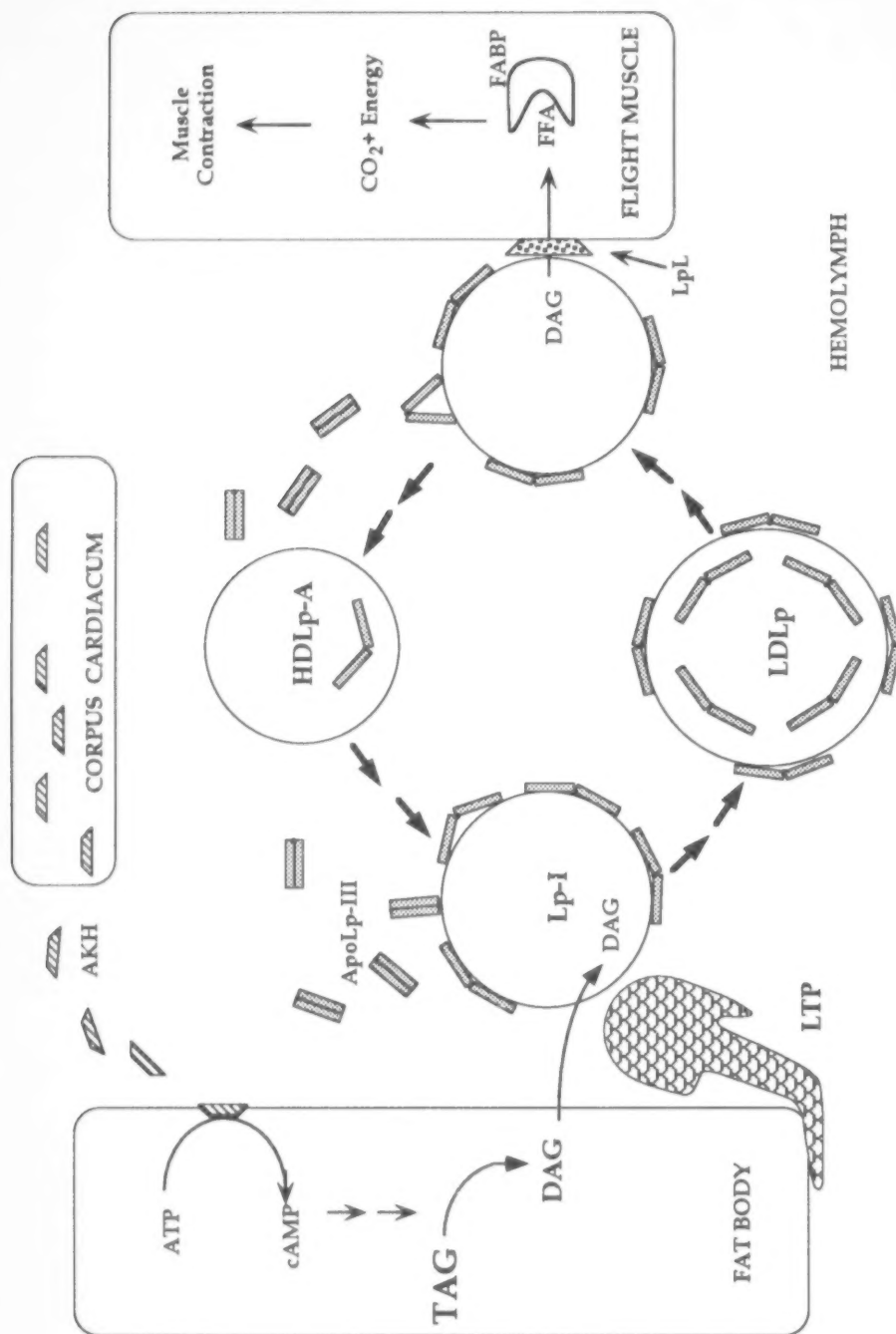
abetalipoproteinemic patients indicating that MTP is involved in apo B-containing lipoprotein assembly and secretion (Sharp *et al.*, 1993).

Parallels between mammalian and insect lipoprotein metabolism exist on a number of levels. The exchangeable apoLp-III may be analogous to apolipoprotein As and Cs. ApoLp-I and apoLp-II appear to be related to apo B in their roles in providing structural stability to neutral lipid-rich lipoproteins and receptor binding. As well, the assembly of insect lipophorins and the recruitment of lipids by LTP has potential to serve as an important model of apo B-containing lipoprotein assembly. The apparent key role of apoLTP-II in the transfer activity of LTP and the analogies in function of MTP and LTP suggest similarities between LTP and the mammalian microsomal triacylglycerol transfer protein complex. The similarity in the subunit size of MTP with apoLTP-II and -III and the identification of apoLTP-II as the catalytically important subunit of LTP is interesting. LTP and MTP can be thought of as carrying out similar functions in the biosynthesis and loading of lipoproteins. LTP is involved in the transfer of lipid from its storage site, the fat body, to lipophorin (Van Heusden and Law, 1989) and may have a role in the transfer of dietary lipids into nascent lipophorin particles at the midgut (Prasad *et al.*, 1986a). Further investigation into the identity of apoLTP-II and -III and their relationship to MTP is ongoing in our laboratory.

This thesis has provided insight into structural characteristics and the functioning of LTP as a lipid transfer catalyst. The model of LTP structure and function described here will serve as a basis for further investigation of the unique properties of LTP and lipid-protein and protein-protein interactions.

Figure 6.1 Revised model of lipid mobilization and transport by lipophorin.

Revised model of the mobilization of lipid stores, transfer of DAG into lipophorin and transport through the hemolymph by lipophorin. LTP transfers DAG into lipophorin utilizing a carrier-mediated mechanism of action in which the lipid-containing head region of LTP moves between the fat body and lipophorin allowing apoLTP-II to facilitate transfer.



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